INTERACTION OF PEPTIDES, PEPTIDOMIMETICS AND OTHER DRUG CANDIDATES WITH THE DI-/TRIPEPTIDE TRANSPORT SYSTEM IN INTESTINAL EPITHELIAL CELLS, USING THE *IN VITRO* CACO-2 CELL CULTURE SYSTEM

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Doctor of Philosophy

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SUMMARY

An uptake system was developed using Caco-2 cell monolayers and the dipeptide, glycyl-[^{3}H]L-proline, as a probe compound. Glycyl-[^{3}H]L-proline uptake was *via* the di-/tri-peptide transport system (DTS) and, exhibited concentration-, pH- and temperature-dependency. Dipeptides inhibited uptake of the probe, and the design of the system allowed competitors to be ranked against one another with respect to affinity for the transporter.

The structural features required to ensure or increase interaction with the DTS were defined by studying the effect of a series of glycyl-L-proline and angiotensin-converting enzyme (ACE)-inhibitor (SQ-29852) analogues on the uptake of the probe. The SQ-29852 structure was divided into six domains (A-F) and competitors were grouped into series depending on structural variations within specific regions. Domain A was found to prefer a hydrophobic function, such as a phenyl group, and was intolerant to positive charges and H^+-acceptors and donors. SQ-29852 analogues were more tolerant of substitutions in the C domain, compared to glycyl-L-proline analogues, suggesting that interactions along the length of the SQ-29852 molecule may override the effects of substitutions in the C domain. SQ-29852 analogues showed a preference for a positive function, such as an amine group in this region, but dipeptide structures favoured an uncharged substitution. Lipophilic substituents in domain D increased affinity of SQ-29852 analogues with the DTS. A similar effect was observed for ACE-NEP inhibitor analogues. Domain E, corresponding to the carboxyl group was found to be tolerant of esterification for SQ-29852 analogues but not for dipeptides. Structural features which may increase interaction for one series of compounds, may not have the same effect for another series, indicating that the presence of multiple recognition sites on a molecule may override the deleterious effect of any one change. Modifying current, poorly absorbed peptidomimetic structures to fit the proposed hypothetical model may improve oral bioavailability by increasing affinity for the DTS.

The stereochemical preference of the transporter was explored using four series of compounds (SQ-29852, lysylproline, alanylproline and alanylalanine enantiomers). The L, L stereochemistry was the preferred conformation for all four series, agreeing with previous studies. However, D, D enantiomers were shown in some cases to be substrates for the DTS, although exhibiting a lower affinity than their L, L counterparts.

All the ACE-inhibitors and β-lactam antibiotics investigated, produced a degree of inhibition of the probe, and thus show some affinity for the DTS. This contrasts with previous reports that found several ACE inhibitors to be absorbed *via* a passive process, thus suggesting that compounds are capable of binding to the transporter site and inhibiting the probe without being translocated into the cell. This was also shown to be the case for oligodeoxynucleotide conjugated to a lipophilic group (vitamin E), and highlights the possibility that other orally administered drug candidates may exert non-specific effects on the DTS and possibly have a nutritional impact.

Molecular modelling of selected ACE-NEP inhibitors revealed that the three carbonyl functions can be oriented in a similar direction, and this conformation was found to exist in a local energy-minimised state, indicating that the carbonyls may possibly be involved in hydrogen-bond formation with the binding site of the DTS.

ACE inhibitors, ACE-NEP inhibitors, structure-activity relationships, SQ-29852,

I dedicate this thesis to my mother and father,
for all their love and support

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CHAPTER ONE

GENERAL INTRODUCTION

ABSTRACT

The general introduction gives an overview into drug absorption studies, focusing on the use of the Caco-2 cell line as an intestinal model for drug absorption studies. The absorption mechanisms of di-/tripeptides and peptidomimetic drugs and oligodeoxynucleotides are reviewed in detail.

1.1 BACKGROUND

Peptides and proteins of potential medicinal importance are now available in large quantities, as a result of the recent developments in the field of biotechnology. Economic viability now allows existing and novel compounds to be considered as therapeutic agents. However, in order to be successful in this role, effective delivery must be achieved.

1.2 PEPTIDE AND PROTEIN AS DRUGS

Peptide and protein drugs may be very potent even at low concentrations, and potentially produce minimal side-effects, thus in many aspects they approach the ideal therapeutic agents [Patel, 1988]. Enzymes, hormones, immunomodulating agents and vaccines are all examples of peptide/protein drugs. In order for these compounds to be medicinally useful, good bioavailability must be achieved. Most therapeutic peptides are administered parenterally, usually by daily injections of aqueous solutions and occasionally as suspensions or solutions *e.g.* insulin. Patient compliance tends to be poor with this method, unless the situation is life-threatening.

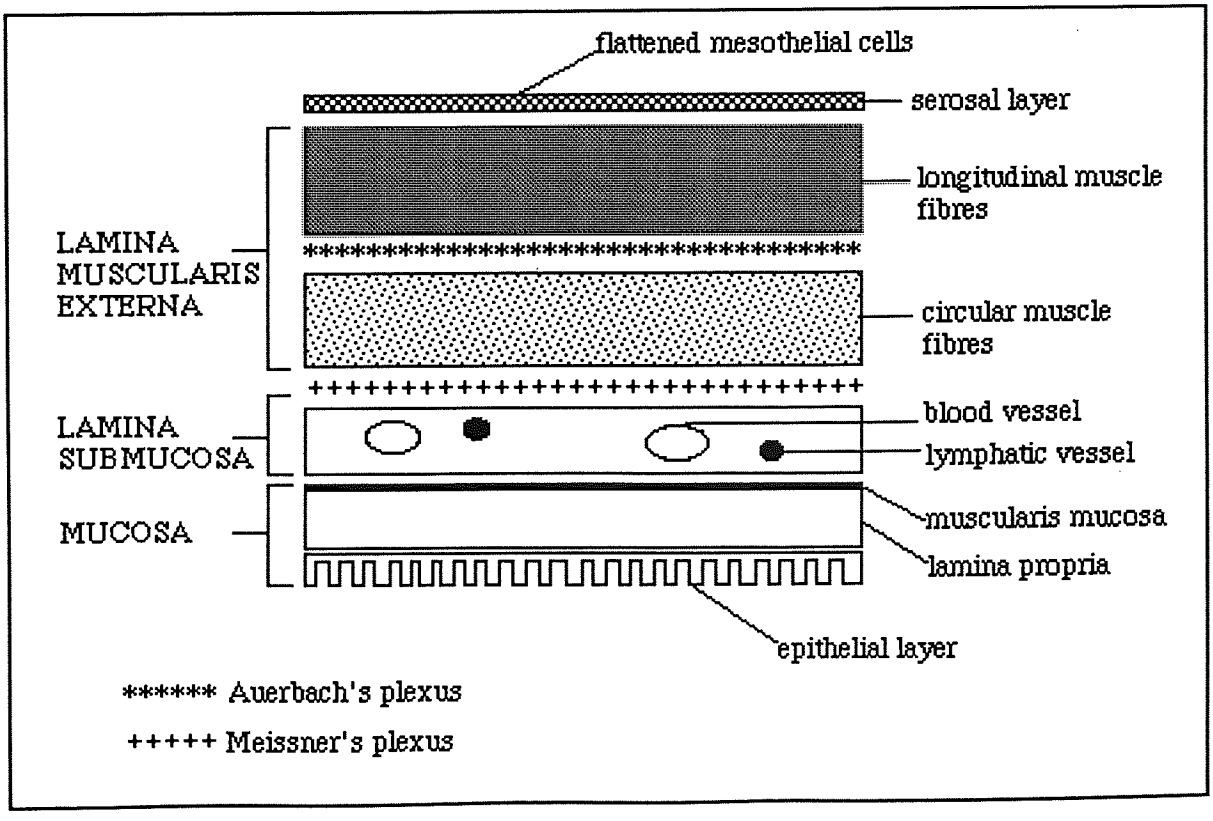
Oral administration is undoubtedly the most favoured route of delivery due to it having good patient compliance, relatively cheap, non-invasive, free from many of the complications that are associated with parental administration, and free from the use of special applicators [Gardner & Wood, 1988]. These advantages only become relevant if sufficiently high bioavailability can be achieved. However, the success of this route is limited due to degradation by dietary enzymes (further discussed in Chapter 3), and poor absorption across epithelial cell membranes of the gastrointestinal (GI) tract, often resulting in oral bioavailabilites of less than 1-2 % [Lee & Yamamoto, 1990]. Peptidomimetics are molecules that mimic the biological action of a peptide, yet are not completely true peptides. Many drug molecules such as angiotensin-converting enzyme (ACE)-inhibitors, β-lactam antibiotics, renin and thrombin inhibitors are peptidomimetics. Due to their peptidic properties, these drugs are largely subject to the same biological and physical barriers a true peptide has to overcome in order to achieve adequate absorption. Therefore, the pharmaceutical industry is extremely interested in improving the absorption of peptidomimetics by modifying current drugs to increase their oral bioavailability, designing new compounds that overcome the barriers to absorption and converting drugs, which have poor bioavailability into peptide prodrugs designed to target the active transport system through which dipeptides are absorbed. To address these problems in order to achieve good oral absorption of peptide and peptidomimetic drugs, the barriers presented by the GI tract, and thus its physiology need to be considered.

1.3 PHYSIOLOGY OF THE GI TRACT

Essentially the GI tract is a hollow tube of approximately 9 m, which is continuous with the external environment, and can be divided into five regions running from mouth to

anus. The general structure is consistent throughout the GI tract with specific modifications in certain regions to facilitate the digestive absorptive process. The digestive tube is composed of four major tissue layers (Figure 1.1). The innermost layer (to the body) is a covering of flattened mesothelial cells with underlying loose connective tissue known as the serosal layer. The serosa secretes a watery fluid which lubricates, preventing friction between the digestive organs and the surrounding viscera. The next layer, the lamina muscularis externa is composed of smooth muscle and makes up the main thickness of the gut. Contractions of the circular and longitudinal muscle fibres in this region are responsible for peristalsis, which propels and mixes the contents of the small intestine. Ganglion cell bodies and nerve trunks of Auerbach's plexus lie between these two muscular regions. The third layer, the lamina submucosa is composed of dense connective tissue, providing elasticity to the GI tract. A second autonomic nerve plexus, the Meissner's plexus, and branching blood and lymphatic vessels are present in this region. The outer most layer is the mucosa, which has three distinct regions, the muscularis mucosa, the lamina propria and the epithelium. The muscularis mucosa is a sparse inner layer of smooth muscle lying adjacent to the lamina submucosa. The lamina propria is a thin middle layer of connective tissue, through which small blood capillaries, lymphatic vessels and nerve fibres pass. It also contains the gut-associated lymphoid tissue (GALT), which is thought to be important in the intestinal defence against invading bacteria. The outermost layer, the epithelium, is the barrier between the external environment (luminal contents) and the body.

FIGURE 1.1 *Structural layers of the GI tract*



The GI tract consists of the mouth, the pharynx and oesophagus, stomach, small intestine and the large intestine. No absorption of foodstuffs takes place in the mouth, the pharynx and oesophagus or the stomach. However, absorption of a few medications such as nitroglycerine takes place in the mouth, and a few lipid-soluble substances such as alcohol and aspirin takes place in the stomach. The small intestine which is the major site for food and drug absorption will be discussed in more detail. The large intestine, consisting of the cecum, colon and rectum, absorbs salts and water, and converts the remaining luminal contents into faeces.

1.3.1 THE SMALL INTESTINE

The small intestine is the main absorptive region for nutrients and drugs along the GI tract. It is made up of the duodenum, the jejunum and the ileum. The length of the duodenum is 0.2 m, while that of the jejunum plus ileum are approximately 2.5 m, in humans [Kararli, 1989]. The architecture of the small intestine is designed to maximise the luminal surface area for optimal absorption. This is achieved by several structural modifications; macroscopic circular folds of the submucosa, the brush-border membrane of the enterocytes (densely packed microvilli) and finally invaginations (crypts of Lieberkuhn) and extensions of the mucosa (villi). The villi, which are 0.5 to 1 mm in diameter, and the microvilli, which are about 1 μm in length and 0.1 μm in diameter [Kararli, 1989] both help to increase the surface area of the small intestine to about 200 m^2 [Houston & Wood 1980].

1.3.2 GI EPITHELIUM

The GI epithelium is composed of a variety of cell types (primarily enterocytes, goblet cells, endocrine cells and Paneth cells) forming a continuous single sheet [Madara & Trier, 1987]. Its task is selective, in that it is required to absorb nutrients and electrolytes yet prevent the uptake of substances such as bacteria, micro-organisms and toxins, which are potentially harmful to the body. The epithelial cells are produced from a single cell population, which is located at the crypts of Lieberkuhn. Cells divide and migrate towards the tips of the villi, differentiating into different cell types on their journey. The average life of an enterocyte is about 3 days (d), and approximately 5×10^7 cells are sloughed off from the extrusion zones of the villi tips each day [Kararli, 1989]. Division and migration of the precursor cells in the crypts is a continuous process, replenishing the daily cell loss, and repairing the damage incurred to the epithelium during the digestive process.

The cells of the GI epithelium are in contact with each other *via* the junctional complexes. Tight junctions are present at the apical region of the cells, below the brush border membrane, forming a tight "seal" between neighbouring epithelial cells [Hochman & Artursson, 1994]. Tight junctions separate the contents of the intestinal lumen from the contents of the intercellular spaces. These limit passive diffusion through the intercellular spaces, therefore controlling the interchange between the luminal contents and the circulation. They also play a role in organisation of the membrane into apical and

basolateral domains. The zonula adherens are proximally situated with respect to the tight junctions, with the intercellular space between cells being increased to 20 nm in this region [Kararli, 1989]. Mechanical stability of the epithelium is provided by the zonula adherens and the macula adherens or desmosomes, which are disk-shaped specialisations [Kararli, 1989].

As previously mentioned the epithelium contains a variety of cells which will be briefly discussed, with the enterocyte or absorptive cells being considered further. Goblet cells are single cup shaped non-absorptive cells found in the crypts. They possess an extensive granular endoplasmic reticulum and an elaborate golgi system conducive to mucus production. Mucus is a complex mixture of glycoproteins, water, serum, cellular macromolecules, electrolytes, microorganisms and sloughed cells [Neutra & Forstner, 1987]. The mucus protects the GI mucosa from acid, pepsin, alcohol and other luminal substances [Neutra & Forstner, 1987]. It also lubricates the surface of the epithelium [Neutra & Forstner, 1987]. M-cells take their name from the microfolds or convolutions on the apical surface [Owen & Jones, 1974]. They over-lie lymphoid tissue associated with the Peyer's Patches and are involved in the stimulation of the immune system. They contain numerous vesicles and have been shown to transport macromolecules [O'Hagan *et al.*, 1987]. Paneth cells, which are pyramidal cells, containing numerous eosinophilic granules at the apical region are found only at the foot of the crypt. They contain immunoglobulin A (IgA) and immunoglobulin G (IgG) [Rodning *et al.*, 1976], and have been shown to be involved in the regulation of the bacterial millieu in the intestine [Satoh *et al.*, 1986]. Defensins are a family of antimicrobial peptides which are thought to contribute to the defence of mucosal surfaces. The expression of human defensin-5 gene in Paneth cells and the localisation of defensin-6 messenger ribonucleic acid (mRNA) to these cells in the crypts of Lieberkuhn has been reported. These findings support the notion that Paneth cells play a role in host defence of the GI tract [Jones & Bevins, 1992, 1993].

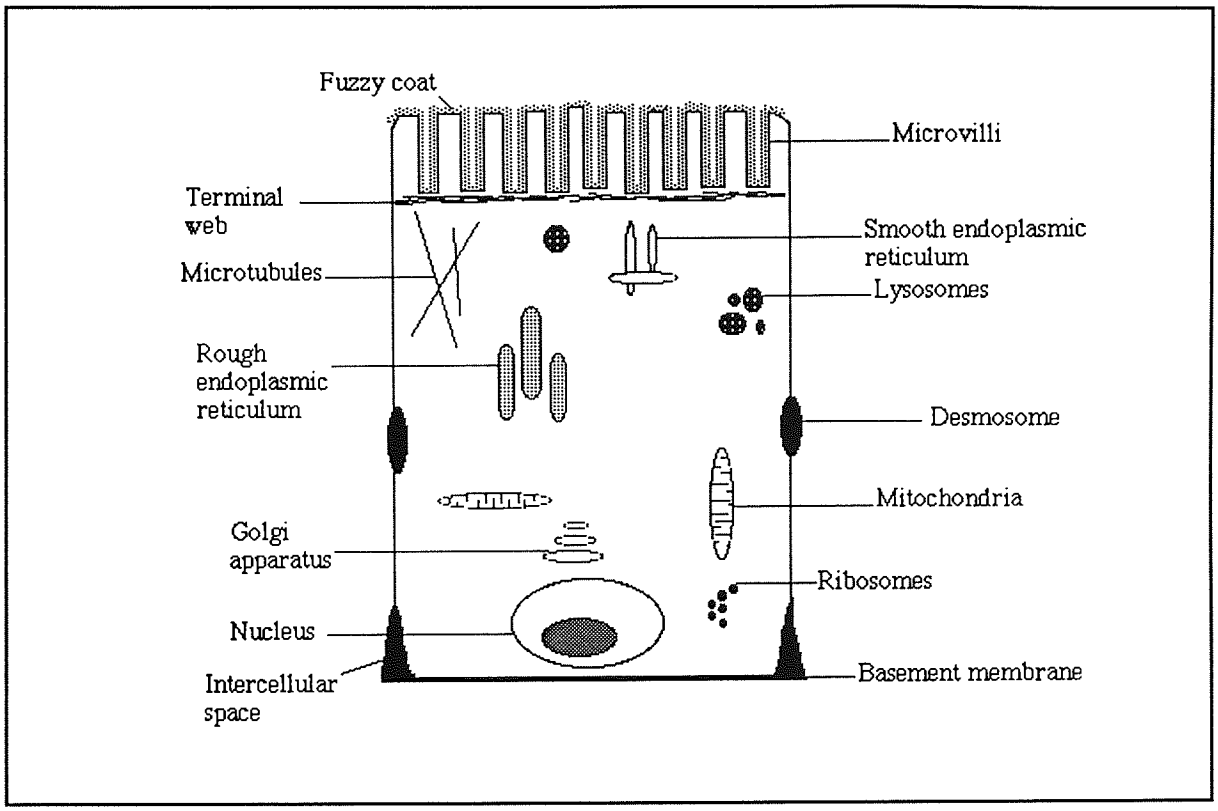
1.3.2.1 ENTEROCYTES

Enterocytes or absorptive cells make up 90 % of the epithelial cell population and are the most important anatomical barrier against drug absorption [Kararli, 1989]. They are produced by maturation of the Crypts of Lieberkuhn cells in the crypts of the villi. Migration of the maturing cells occurs along the length of the villi, and they are eventually sloughed off at the tips into the lumen, after about 3 d [Kararli, 1989]. The apical or brush border membrane which is densely packed with microvilli, is their most distinctive feature (Figure 1.2). It has a glycocalyx or fuzzy coat, which is a glycoprotein produced by the enterocyte extending 0.1 μm from the microvilli tips [Ito, 1974], aiding in the digestive process (contains digestive enzymes) and protecting the intestinal mucosa.

The enterocyte has a typical volume of about 250 μm^3, containing a nucleus, golgi apparatus, and rough and smooth endoplasmic reticulum which play a role in transferring the

absorbed lipids into lymph vessels [Yih-Fu, 1981]. Mitochondria, microtubules, lysosomes and cytoplasmic filaments are also present.

FIGURE 1.2 *Schematic diagram of the enterocyte (adapted from Kararli, 1989)*



1.4 TRANSEPITHELIAL TRANSPORT

A permeant (drug molecule) may cross from the intestinal lumen into the circulation or the lymphatic system *via* two major pathways, the paracellular and the transcellular route (Figure 1.3). The paracellular route involves diffusion through the junctional complexes or the transient gaps between cells at the extrusion zones on the villus tips, while the transcellular route involves the movement of a permeant through the epithelial cell. The paracellular route exclusively involves passive transport, whereas the transcellular route can incorporate both passive and active transport.

Passive transport refers to the movement (diffusion) of a solute along its concentration and electrical gradient. The chemical potential difference between the solute concentration on either side of the membrane provides the driving force for the diffusion of the molecule through the aqueous channel [Kararli, 1989]. Passive transport can occur *via* diffusion through aqueous channels in the membrane and *via* the tight junctional complexes. The diffusion of molecules with molecular weights of less than 180 dalton (Da) have been suggested *via* the aqueous pores [Kingham & Loehry, 1976], while absorption of small hydrophilic and charged molecules has been shown to occur *via* the junctional complexes [Artursson, 1991], with hydrophobic drugs going *via* the transcellular route.

Lipid-partitioning is another form of passive transport (transcellular) and involves the incorporation of a hydrophobic drug molecule into the lipid bilayer (membrane) of the cell and then partitioning of the molecule into the cell. The lipid bilayer is highly impermeable to charged molecules, as the charge and high degree of hydration prevents them from entering the hydrocarbon phase of the bilayer. The diffusion of hydrophobic drugs through the unstirred water layer (UWL) or aqueous boundary layer (ABL) is the rate-limiting step in this process. The absorption rate of drugs through biological membranes is directly proportional to the value of the oil/water partition coefficient. At low Log P values (logarithm of the partition coefficient) *e.g.* -2.46 for thyrotrophin-releasing hormone, (TRH) [Bundgaard & Moss, 1990] a drug can not penetrate the lipid membrane. However, at high Log P values, the drug becomes so lipophilic that diffusion through the UWL will become the rate-limiting step for absorption. A study using two intestinal cell culture models, HT29-18-C_1 and Caco-2 cells found that when the octanol/buffer distribution coefficient of drugs ($D_{o/b}$) were lower than 3.5, the transepithelial coefficient increased [Wils *et al.*, 1994]. However, when log $D_{o/b}$ values were between 3.5-5.2 the transepithelial coefficient decreased with increasing lipophilicity as suggested previously [Wils *et al.*, 1994].

Facilitated diffusion involves the reversible binding of specific substrates to proteins in the cell membrane to facilitate (assist) the transport of a substance across the membrane, downhill from high to low concentration. The process is saturable, but no energy is required seeing that transport does not occur against a concentration gradient [Kararli, 1989].

Active transport also involves the binding of specific substrates to membrane-located proteins. However, it differs from facilitated diffusion as transport occurs against a concentration gradient and therefore, requires energy [Kararli, 1989]. The energy can be supplied in one of two ways, either involving a coupled transport process whereby a second substrate is transported (symport or antiport) down its concentration gradient releasing energy (*e.g.* glucose and amino acid symport system with Na^+). The second alternative is where the substrate binds to a transporter protein (pump), with energy being released from the hydrolysis of adenosine triphosphate (ATP). The transporter protein is then phosphorylated resulting in a conformational change in the pump bringing the active site with its ligand to the other side of the membrane and thus translocation into the cell (*e.g.* Na^+ by Na^+, K^+ ATPase) [Kararli, 1989]. Active transport processes are inhibitable by metabolic inhibitors, such as 2, 4-dinitrophenol (DNP) and sodium azide and require molecular oxygen. Substrate analogues of the transported compound can also inhibit transport by competing for the active site on the transporter. The temperature gradient is also higher than that for passive processes [Kararli, 1989].

Another form of transport is receptor-mediated transcytosis involving the binding of membrane located receptors to macromolecules which are then transported across the cell in endocytosed vesicles [Artursson, 1991]. Cholesterol is transported into cells by binding to low density lipoprotein (LDL) at the apical membrane and is then transported *via* endocytosed vesicles. Fluid-phase endocytosis involves the incorporation of extracellular

fluid which contains dissolved substances into the endocytosed vesicle, which is then transported through the cell.

Most drugs that are given orally are lipophilic, having a high membrane permeability due to lipid-partitioning, and are therefore, transported by the transcellular route, *e.g.* corticosterone, testosterone and propranolol. Hydrophilic (*e.g.* terbutaline and atenolol) and charged drugs are absorbed *via* the paracellular route, as they are impermeable to the cell membrane. The GI epithelia cells has a number of active and facilitated transporter systems which are involved in the absorption of various substances across the cell barrier. The apical systems which are involved in the transport of drug compounds are shown in Table 1.1 [Tsuji & Tamai, 1996]. The epithelial cells also express a number of other transporters which are involved in the absorption of nutrients and ions across the GI tract; various amino acid transport systems (*e.g.* B, $B^{0,+}$, $b^{0,+}$, y^+, IMINO, β, and X^-_{AG}), carbohydrate transport systems (*e.g.* D-fructose and Na^+-dependent D-glucose), H^+-dependent lactic acid and short-chain fatty acid transporters, HCO_3^--dependent and a H^+-dependent nicotinic acid transporter, OH^--dependent folic acid transporter, a choline transport system and an Na^+/H^+ antiporter which is involved in the regulation of a H^+ gradient across the membrane [Tsuji & Tamai, 1996].

The di-/tripeptide transporter, which has been referred to as the oligopeptide transporter in previous studies [Hidalgo *et al.*, 1995], will, throughout this report be referred to as the DTS. The DTS, which is involved in the transport of di-/tripeptides and peptidomimetic drugs will be discussed in further detail (section 1.7).

FIGURE 1.3 *Transepithelial transport (adapted from Artursson, 1991)*

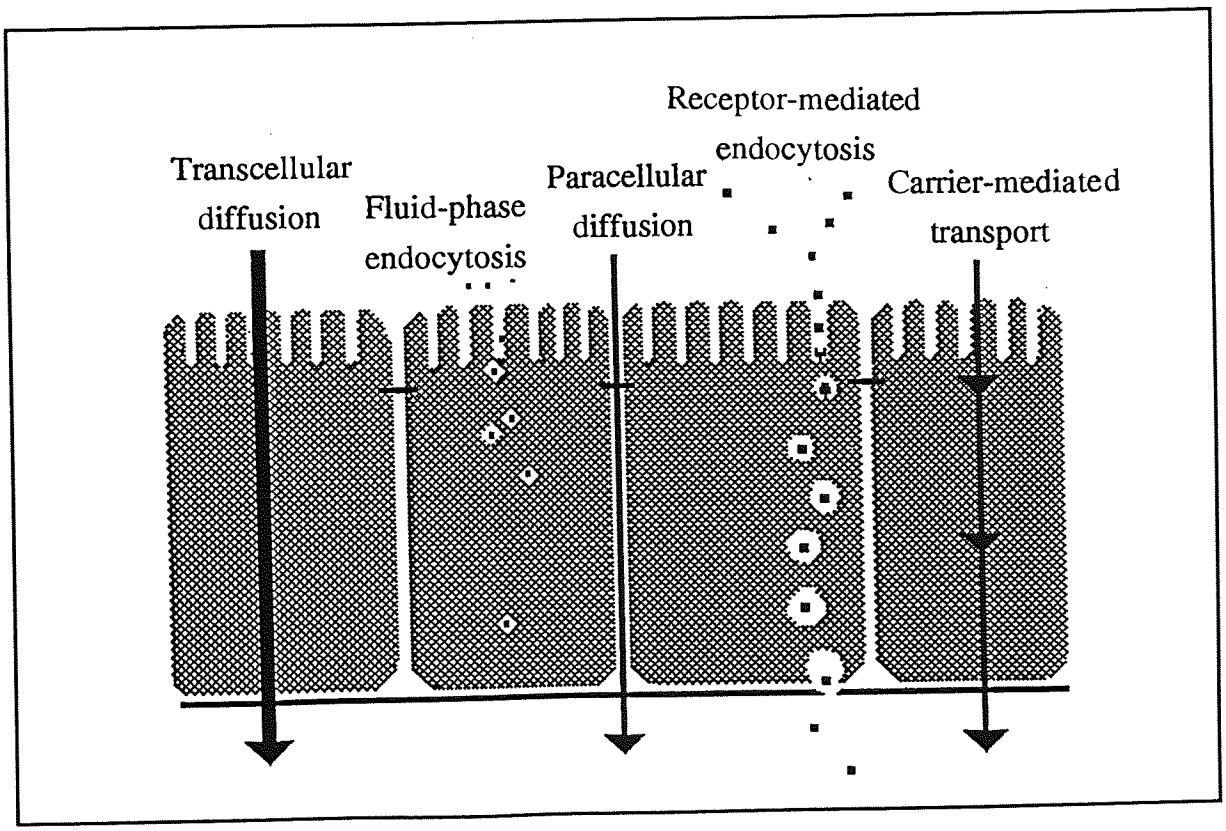
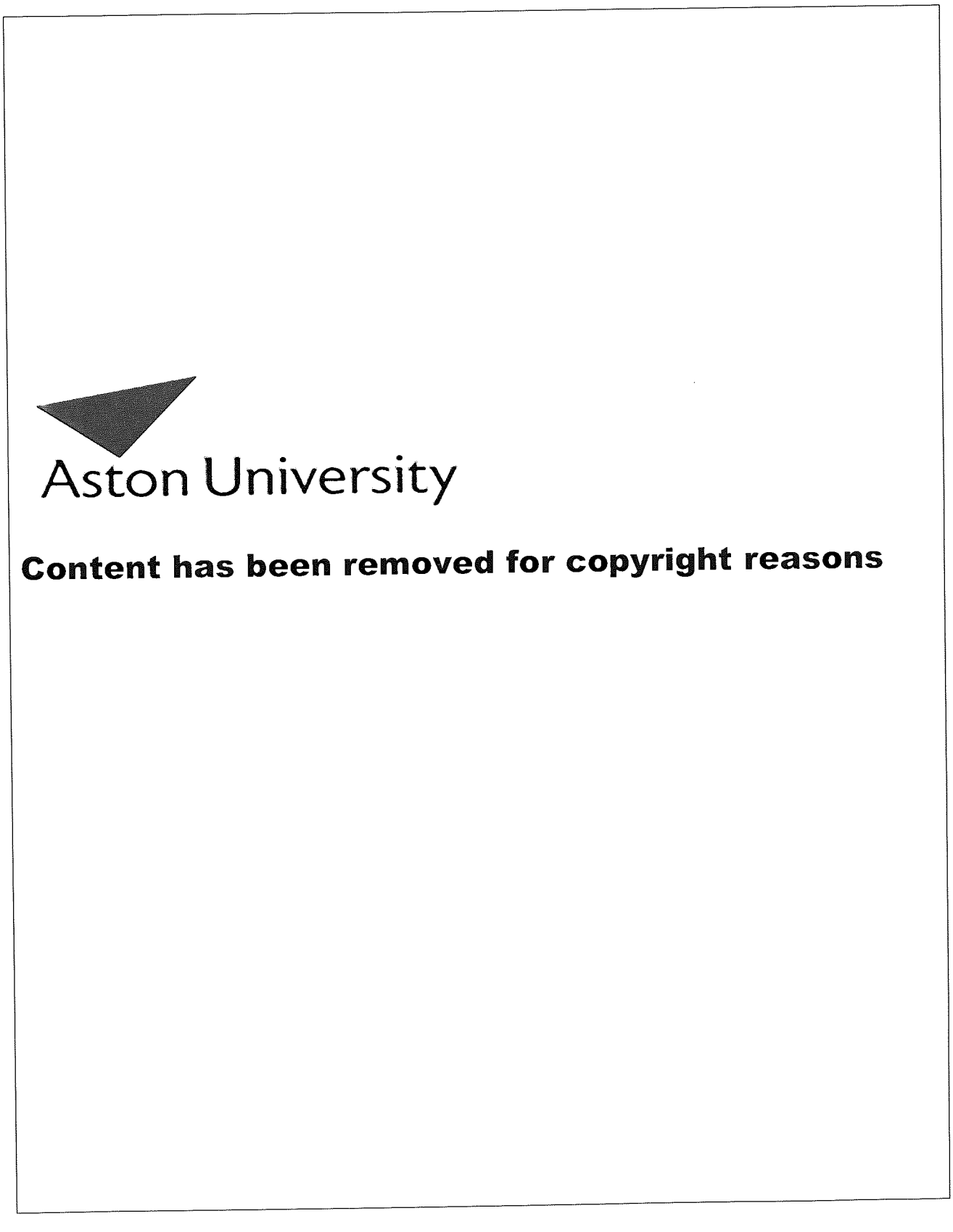


TABLE 1.1 *Summary of the apical transporter systems expressed by intestinal epithelial cells, which are involved in drug transport [Tsuji & Tamai, 1996]*



1.4.1 KINETICS OF ACTIVE TRANSPORT

The binding of a transporter protein to its substrate resembles that of an enzyme-substrate reaction, and therefore the kinetics of the process follow the same principles. When the transporter protein is saturated, the rate of uptake/transport is maximal, this is referred to as V_{max} or J_{max} The carrier also has a characteristic binding constant for the substrate, this is referred to as the Michaelis constant (K_m), which is equivalent to the concentration of the substrate when uptake/transport is half its maximum value. Some previous studies have used the term K_t to refer to the half-maximal transport concentration (K_m) [Gochoco *et al.*, 1994: Swaan *et al.*, 1995]. The relationship of the reaction follows the Michaelis-Menten model, shown in Equation 1.1:

EQUATION 1.1

$$V_0 = \frac{V_{max}[S]}{K_m + [S]}$$

where K_m is Michaelis (affinity) constant , V_{max} is the maximal uptake/transport rate and S is the initial substrate concentration.

The uptake /transport of a compound across the GI tract is very rarely 100 % carrier-mediated, and a passive diffusional component is present. Therefore, the equation 1.1 is modified to incorporate this (Equation 1.2):

EQUATION 1.2

$$V_0 = \frac{V_{max}[S]}{K_m + [S]} + k_d[S]$$

where k_d is the rate constant of the non-saturable component (passive transport).

The transport of a substrate by the transporter protein can be subject to inhibition by another compound, which can either be competitive or non-competitive. Competitive inhibitors compete with the substrate for the active site of the transporter protein. A competitive inhibitor combines reversibly with the active site, and therefore, inhibition can be reversed by increasing the substrate concentration. In the presence of a competitive inhibitor the V_{max} is unchanged, whereas the apparent K_m is increased to a value of:

EQUATION 1.3

$$\text{apparent } K_m = 1 + \frac{[I]}{K_m}$$

where K_i is the inhibitor dissociation constant of the transporter inhibitor complex. K_i is a measurement of affinity of the transporter for the inhibitor, in the same sense as K_m is a

measure of the affinity for the substrate, therefore the K_m of a substrate for the transporter equals the K_i when the substrate is an inhibiting compound (*e.g.* $K_m = K_i$). K_i is the uptake/transport inhibition constant, which is related to inhibition of carrier-mediated uptake in section 3.3.2, equation 3.3. Throughout the thesis results are expressed as IC_{50} values, which are the same as K_i values, when assuming competitive inhibition at low substrate concentrations. The derivitisation is shown in section 3.2.2, equation 3.4.

1.5 BARRIERS TO DRUG ABSORPTION

Factors affecting the bioavailability of drugs falls into three categories; physiological factors, physicochemical properties of the drug, and dosage form factors. The physiological barrier presented by the GI tract will be considered further. Transport of peptidomimetic drugs across the GI epithelial cells is only viable if the biological and physical barriers presented by the GI tract can be overcome. The intestinal epithelium, which is the interface between the environment and the body, not only acts as a physical barrier by restricting the movement of molecules along the paracellular and transcellular route, but also as a biochemical barrier due to metabolism *via* enzyme systems and transport back into the GI lumen *via* efflux systems [Pauletti *et al.*, 1996]. The metabolic barrier, which results in the enzymatic degradation of proteins and peptides will be discussed in more detail in Chapter 3.

The epithelium is a critical diffusional barrier for the absorption of water-soluble drugs, which as previously mentioned can occur *via* the aqueous pores or tight junctions if of the appropriate molecular weight and size. The UWL and mucus layer are diffusional barriers to highly lipophilic drugs, as discussed previously. Binding and electrostatic repulsion are obstacles which are also associated with the UWL, which have been shown to be barriers to drug and nutrient absorption [Barry & Diamond, 1984]. Drugs are not only subject to enzymatic degradation in the GI lumen, at the brush-border membrane, and in the cytosol, but also in the blood and the liver .

Minimal drug absorption occurs in the stomach, however, it does influence drug absorption in a number of ways. Firstly, the pH of the stomach can fall as low as 2 and, at this extremity, degradation may occur not only as a result of acidic conditions, but also enzymatic attack. Secondly, binding of drug molecules to the luminal contents also takes place. Thirdly, gastric emptying will significantly alter the absorption of drug molecules in the intestine [Kararli, 1989]. After a meal, the residence time of particulates > 2 mm in the stomach will increase, whereas those that are < 2mm are unaffected by the presence food [Davis *et al.*, 1986].

The pH of the GI tract is an important factor when considering drug absorption. As previously mentioned, the harsh extremity of the stomach pH may possibly degrade drugs, and the changing pH values throughout the GI tract will affect the absorption of certain drugs. Decreasing pH values favour the formation of unionised species for weak acids and

ionised species for weak bases, therefore the absorption of weak acids may be expected to increase with lowering pH. However, for peptidomimetic drugs, such as the cephalosporins and the ACE-inhibitors, which have been shown to ultise the DTS, values above or below pH 6 will affect the functioning of the transporter. The presence of an acidic microclimate adjacent to the intestinal mucosa has been reported [Lucas *et al.*, 1975]. This is a region of approximately 20 μm thick, with a pH value 0.5 lower than that of the lumen [Lucas *et al.*, 1975]. Although the function of the acidic microclimate has not been fully documented, it will affect absorption by altering the fraction of ionised drug at the absorption site [Kararli, 1989].

Bile fluid (salts) also has implications in drug absorption. Bile salts have been shown to improve the dissolution rate and equilibrium solubility of water insoluble drugs (*e.g.* cholesterol [Montet *et al.*, 1979]) and thus enhance drug absorption [Kararli, 1989].

1.6 MODELS FOR DRUG ABSORPTION STUDIES

Using normal human tissue for drug absorption studies has its limitations due to limited availability and poor viability upon excision [Trier, 1980]. Numerous methods are available for investigating internal absorption mechanism and for assessing oral bioavailabilities of drug compounds.

1.6.1 *IN VIVO*

In vivo techniques involve the use of intact unanaesthetised animals. The absolute bioavailabilities of drug formulations are determined after oral and intravenous dosing [Kararli, 1989]. These studies allow pharmacists to optimise dissolution and formulation parameters, usually by *in vitro* tests, in order to achieve the most suitable pharmacokinetic profile and pharmacological effects. The effects on drug absorption of pH, peristalsis in the GI tract and the presence of food in the stomach and intestine can be assessed. Methods in this category include the Thiry-Vella Fistula method, monitoring blood levels after administration of the drug into the intestinal lumen, and the Cori method [Csaky, 1984].

1.6.2 *IN SITU*

The animal is anaesthetised and surgically manipulated but the mesenteric blood flow is intact, therefore non-physiological diffusion of the drug through the submucosal and muscularis layer is not a factor to be considered [Kararli, 1989]. The procedure involves a midline incision which is made to expose the small intestine. The region under investigation is then drawn to the surface, where it can either be an open- or closed-loop system, with the drug being introduced at this point. The open-loop system involves recirculating of the drug solution by a pump mechanism after periodic sampling. A three-way syringe is used in the closed-loop system, which is used to introduce the drug and take samples at the relevant time points. The remaining drug solution after sampling, is reintroduced into the intestinal

segment within a 30 second (sec) period. The drawbacks of this system are that binding and metabolism of the drug can occur in the mucosal tissue, therefore, the disappearance of the drug from the luminal contents may not be a true representation of the appearance rate in the blood. The volume of the luminal contents is not a constant value due to absorption and secretion of water, resulting in an alteration of luminal drug concentration. Phenol red, polyethylene (PEG) $_{4000}$ and insulin, which are nonabsorbable marker compounds can be used to compensate and monitor the variation in the volume of the luminal contents [Miller *et al.*, 1970].

1.6.3 *IN VITRO*

Numerous *in vitro* methods are available for the study of drug absorption. These include everted intestinal preparations, everted intestinal ring slices, brush-border membrane vesicles (BBMV), isolated membranes of mucosal cells and isolated enterocytes. Cell culture models fall into the *in vitro* category and will be considered in more detail.

1.6.3.1 CELL CULTURE MODELS

Cell culture models have a number of advantages over conventional techniques previously used to study drug absorption:

1) Epithelial permeability and metabolism of a drug can be rapidly assessed,
2) They provide the opportunity to study mechanisms of drug transport or pathways of degradation (or activation under controlled conditions),
3) Rapid evaluation of methods to enhance drug absorption, by achieving drug targeting, enhancing drug transport and minimising drug metabolism,
4) Opportunity to perform studies on human cells,
5) Reduction in the time consuming, expensive and often controversial animal experiments [Audus *et al.*, 1990].

Human GI epithelial cells are the most suitable and comparable cells to assess transport of drugs across the GI tract, however, they are not ideal as cell culture models. The cells are easily obtained, but do not form viable primary cultures, and fail to proliferate and re-establish monolayers. They grow as islands of cells consisting of centrally dividing regions and external differentiated areas [Moyer, 1983].

Alternative approaches using human adenocarcinoma cell lines have been investigated. Cancer cells show a varying degree of differentiation, and only cell lines which resemble intestinal enterocytes, forming polarised monolayers with well-developed barrier properties, are suitable for drug absorption studies [Artursson, 1991]. The cell lines are classified depending on their degree of differentiation into four categories. Type 1 and 2 will be discussed further. Type 3 cells (*e.g.* HCT-GLY, HCT-FET) although displaying organised monolayers with dome formation, lack characteristics of enterocytic differentiation whatever the culture conditions. Type 4 cells are organised into a multilayer

without any feature of epithelial polarity or enterocytic differentiation [Chantret *et al.*, 1988].

Caco-2 and HT29 are the most thoroughly investigated human colon carcinoma cell lines due to their morphologic features resembling those of mature enterocytes. Both these cell lines produce villin, a Ca^{2+}-regulated actin-binding protein which is specifically associated with the cytoskeleton of brush border microvilli [Robine *et al.*, 1985]. The HT29 cell line belongs to the Type 2 category, which do not spontaneously differentiate. However, changing the culture conditions from glucose to galactose induces differentiation [Zweibaum, 1985].

The Type 1 category, for which the Caco-2 cell line is the only member, undergoes spontaneous differentiation under normal cell culture conditions forming polarised monolayers and well-developed apical brush borders with several hydrolases. The Caco-2 cell line is the most favoured cell culture model due to easy cultivation and a higher degree of differentiation than Type 2 cell lines [Chantret *et al.*, 1988]. Sucrase-isomaltase, lactose-phlorizin hydrolase and aminopeptidase *N* are expressed by Caco-2 cells [Hauri *et al.*, 1985]. The peptide metabolising enzyme dipeptidylpeptidase IV, is also expressed in a higher concentration than in Type 2 cell lines [Yoshioka *et al.*, 1991], making the Caco-2 cell line the most appropriate model.

Absorptive cells, such as Caco-2 cells which are used for drug absorption studies, produce mucin molecules but do not establish a visible mucus layer [Wikman *et al.*, 1993]. The mucus layer which is a barrier to diffusional drug absorption, is produced by the goblet cells which are found as single cells in the crypts of the villi. The absorption of compounds is directly proportional to the diffusion through the goblet-cell mucus and inversely proportional to retention in the mucus, with the molecular weight of the compound also affecting diffusion [Nimmerfall & Rosenthaler, 1980]. Several goblet cell lines have recently been established from HT29 [Phillips *et al.*, 1988]. The clones (HT29-H) differentiate to produce monolayers that contains a large proportion of mature goblet cells [Phillips *et al.*, 1988: Kreusel *et al.*, 1991]. HT29-H cells have been shown to form monolayers of mature goblet cells under standard conditions and secrete mucin molecules which form a mucus layer covering the apical membrane of the cell [Wikman *et al.*, 1993]. The mucus layer has also been shown to be a significant barrier to the absorption of testosterone, which is a lipophilic drug [Wikman *et al.*, 1993]. HT29-H monolayers offer a unique model for studying the effect of the mucus layer on drug and peptide absorption under controlled cell culture conditions.

1.7 PEPTIDE TRANSPORT

1.7.1 INTRODUCTION

It was believed for many years that proteins were hydrolysed into their constituent free amino acids before absorption across the intestinal epithelium *via* the relevant amino acid carriers. However, in 1968, absorption of intact dipeptides into the portal vein was observed, with greater absorption of amino acids arising from administration of the dipeptide rather than the free amino acid in the human intestine, suggesting absorption of intact dipeptide [Adibi & Phillips, 1968]. The distinction between amino acid and dipeptide transport is reinforced by the following pieces of evidence;

Firstly, studies in patients with Hartnup's disease, which is a condition whereby the carrier mechanism for neutral amino acids (*e.g.* L-Phe and L-tyrosine {L-Tyr}) is grossly inadequate, were carried out. If the "affected" amino acid is presented to the mucosa in the dipeptide form, absorption is intact, indicating the presence of dipeptide absorption [Asatoor *et al.,* 1970]. Similar results were found in patients with cystinuria, a condition where the absorption of cysteine (Cys) is impaired [Hellier *et al.*, 1972].

Secondly, studies investigating dipeptide transport across the intestinal epithelia of rabbit ileum, showed that dipeptides do not compete with their constituent amino acids in the free form for transport, whereas dipeptides do compete with one another [Rubino *et al.*, 1971]. Unhydrolysed glycylglycine (Gly-Gly) was detected in the peripheral plasma during perfusion of the small intestine of man, indicating disappearance is accomplished exclusively by intact dipeptide absorption [Adibi, 1971].

Ontogenetic evidence for the distinction between the dipeptide and amino acid transporter has also been provided. Uptake of 0.5 mM glycyl-L-proline (Gly-L-Pro) into the jejunum and ileum of rabbits from the 25^{th} d of gestational age into adulthood was compared to uptake of 0.5 mM glycine (Gly) [Guandalini & Rubino, 1982]. Influx of Gly-L-Pro was present from the 25^{th} d of gestation showing a steep prenatal increase which peaked at birth. Maximum velocity in the newborn was 45.2 ± 3.3 mol g h^{-1}, which decreased to 3.8 ± 0.5 mol g h^{-1} in the adult [Guandalini & Rubino, 1982]. Gly uptake showed no change throughout the period studied. This data illustrates that there is a very efficient dipeptide transport system in the new born which shares characteristics with that described in the adult. The developmental patterns for the two transporters are different, indicating a distinction between them [Guandalini & Rubino, 1982].

1.7.2 DIPEPTIDE ABSORPTION

The evidence for active carrier-mediated dipeptide transport is persuasive. Two main processes contribute to peptide absorption; transport of amino acids liberated by complete hydrolysis, by active amino acid transport systems, and uptake of dipeptides from partial hydrolysis, by the DTS [Silk, 1981]. Accounts of absorption *via* the DTS and the characteristics shown are outlined below;

Glycylsarcosine (Gly-Sar) was found to be transported into hamster jejunal mucosal cells by an active Na^+-dependent process [Addison *et al.*, 1972]. L-Methionyl-L-methionine (L-Met-L-Met) inhibited Gly-Sar uptake, whereas L-methionine (L-Met) was without effect [Addison *et al.*, 1972]. These results suggest that Gly-Sar and L-Met-L-Met may share the same transport system and that it is independent of the amino acid (L-Met) transport.

Incubating rings of everted hamster jejunum with carnosine (β-alanyl-L-histidine, {β-Ala-L-His}) resulted in large amounts of the dipeptide appearing in the intestinal wall, accompanied by small amount of the constituent amino acids, which resulted almost entirely from intracellular hydrolysis of the dipeptide [Matthews *et al.*, 1974]. Uptake was saturable, Na^+-dependent, and reduced by anoxia and metabolic inhibitors, suggesting a carrier-mediated process [Matthews *et al.*, 1974].

Studies investigating glycyl-L-leucine (Gly-L-Leu) intestinal transport in monkeys and man, indicated that the dipeptide is transported as one unit [Das & Radhakrishnan, 1974]. Gly-L-Leu uptake was inhibited by a wide variety of dipeptides, including those containing acidic and basic amino acids in the monkey intestine [Das & Radhakrishnan, 1974]. The inhibition was shown to be competitive, indicating that the monkey intestine expresses a dipeptide uptake system with an extremely broad specificity [Das & Radhakrishnan, 1974].

Studies using rabbit intestinal BBMV showed that Gly-L-Pro was transported as the intact dipeptide, illustrating that the hydrolysis of the dipeptide does not precede transport [Ganapathy *et al.*, 1981]. L-Alanyl-L-proline (L-Ala-L-Pro) competed with Gly-L-Pro for transport and also for hydrolysis [Ganapathy *et al.*, 1981]. Papain digestion of the BBMV resulted in a 40 % increase in Gly-L-Pro transport, whereas it caused a 60 % reduction in the transport of Na^+-dependent L-Ala. The increase in transport of the dipeptide is probably a result of a decrease in the thickness of the unstirred water layer adjacent to the brush border. Inhibition of Gly-L-Pro transport by a number of dipeptides was seen in papain treated BBMV, both in the presence and absence of a Na^+ gradient. This indicates that the dipeptide transport in the papain treated vesicles is a Na^+-independent, carrier-mediated process *via* the DTS, as in the control vesicles [Ganapathy *et al.*, 1981].

The absorption of glutamic acid (L-Glu)-dipeptides in the human intestine was investigated using L-alanyl-L-glutamate (L-Ala-L-Glu) and glycyl-L-glutamate (Gly-L-Glu). Hydrolysis rates of the two dipeptides at the brush border membrane were threefold greater for L-Ala-L-Glu than Gly-L-Glu [Minami *et al.*, 1992]. Peptide-bound L-Glu was unaffected in the absence of Na^+ and free amino acids, but inhibited in the presence of di- and tripeptides [Minami *et al.*, 1992]. The presence of a H^+ gradient stimulated uptake, indicating that L-Glu-dipeptides are absorbed as intact dipeptides *via* the DTS, rather than hydrolysed into amino acids and then absorbed [Minami *et al.*, 1992].

1.7.3 TRIPEPTIDE ABSORPTION

The DTS has been shown to be capable of taking up tripeptides as well as dipeptides, but no capacity for transporting tetrapeptides has been observed. The tripeptides glycyl-sarcosylsarcosine (Gly-Sar-Sar) and β-alanylglycylglycine (β-Ala-Gly-Gly) which are resistant to hydrolysis, were found to be taken up intact by everted rings of hamster jejunum [Addison *et al.*, 1975b]. Uptake of Gly-Sar-Sar was a saturable process, with accumulation in the intestinal wall against an electrochemical gradient [Addison *et al.*, 1975b]. Anoxia, metabolic inhibitors and Na^+ replacement reduced uptake, indicating an active Na^+-dependent mechanism [Addison *et al.*, 1975b]. Di- and tripeptides inhibited uptake of the two tripeptides whereas amino acids were without effect, suggesting that tripeptides share the uptake mechanism for dipeptides [Addison *et al.*, 1975a, b]. The tetrapeptide glycylsarcosylsarcosylsarcosine (Gly-Sar-Sar-Sar) was very poorly taken up [Addison *et al.*, 1975b] did not inhibit uptake of Gly-Sar-Sar, indicating that tetrapeptides are unable to utilise the uptake mechanism [Addison *et al.*, 1975b].

The protein (PepT1) involved in dipeptide H^+ coupled transport in rabbit intestine has recently been cloned [Fei *et al.*, 1994]. PepT1 has been expressed in *Xenopus laevis* oocytes, which were shown to take up dipeptides [Fei *et al.*, 1994]. The optimal length of oligopeptides preferred by PepT1 was investigated using oligomers of Gly (Gly_n: n =1-5), by measuring the change in current resulting from peptide/H^+ cotransport. The current was large for the Gly dimer, but decreased when n >2 [Fei *et al.*, 1994]. Larger peptides (n = 5-10) produced no significant change in current, indicating that they are not compatible with the transporter [Fei *et al.*, 1994].

1.7.4 CHARACTERISTICS OF THE DTS

1.7.4.1 THE DI-/TRIPEPTIDE/H^+ COTRANSPORT SYSTEM

Di-/tripeptide transport in a variety of tissue preparations has been shown to be inhibited by anoxia and metabolic inhibitors, *e.g.* cyanide and dinitrophenol, suggesting an active, energy requiring process. It was originally thought that peptide transport was energised by a Na^+ gradient, similar to the transport of amino acids and sugars [Rajendran *et al.*, 1987]. However, the transport/uptake of several dipeptide compounds has been shown to be stimulated in the presence of an H^+ gradient, and it is now well accepted that dipeptide transport is *via* a Na^+-independent H^+ cotransport system.

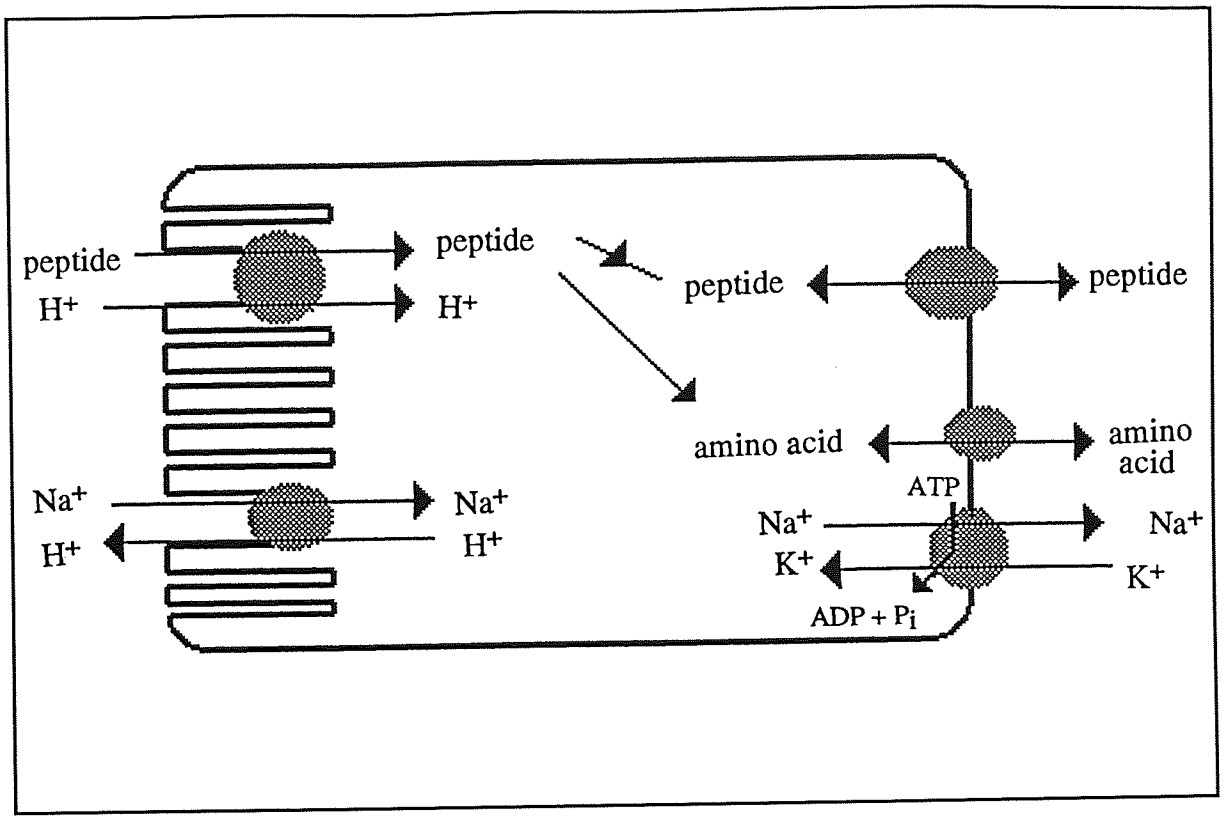
The uptake of Gly-L-Pro and Gly-L-Leu were studied using everted rings from rat small intestine; 50 % of Gly-L-Leu was found to undergo superficial hydrolysis before being absorbed into the rings [Cheeseman & Johnston, 1982]. Experiments using excess free L-leucine (L-Leu) showed a 50 % reduction in Gly-L-Leu uptake. In order to investigate the role of Na^+ in dipeptide absorption, the L-Leu uptake must be blocked, which was achieved by 20 mM free L-Leu . Under these conditions the replacement of Na^+ with choline had no

effect on the uptake of the dipeptide, indicating that the peptide transport does not require Na^+ [Cheesman & Johnston, 1982].

Gly-L-Pro transport into rabbit intestinal and kidney BBMV was found to be optimal when the extravesicular pH was 5.5-6.0 [Ganapathy & Leibach, 1983]. At this pH, Gly-L-Pro transport was accelerated 2-fold by the presence of an inward H^+ gradient [Ganapathy & Leibach, 1983]. A valinomycin-induced K^+ diffusion potential (interior-negative) stimulated Gly-L-Pro transport in Na^+ and Na^+-free conditions [Ganapathy & Leibach, 1983]. When a H^+ diffusion potential (interior-positive) was induced by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), Gly-L-Pro transport was reduced [Ganapathy & Leibach, 1983]. The present study suggested that Gly-L-Pro and H^+ are cotransported, with the process involving a net transfer of positive charge into the cell [Ganapathy & Leibach, 1983]. Harmalin, a compound which competes with Na^+ for the binding site on Na^+-dependent transport systems had no effect on Gly-L-Pro transport in BBMV, indicating that Gly-L-Pro is taken up at the brush-border membrane by a Na^+-independent process [Ganapathy *et al.*, 1981]. Harmaline may effect the transport in intact tissues as it effects the Na^+-K^+-ATPase at the basolateral membrane.

Ganapathy & Leibach, have proposed a hypothesis explaining dipeptide transport with respect to H^+ and Na^+ (Figure 1.4) [Ganapathy & Leibach, 1985]. Dipeptides are cotransported with a H^+, resulting in a depolarisation of the brush border membrane [Boyd & Ward, 1982]. Peptide transport is enhanced with an interior negative membrane potential and inhibited by an interior positive potential. There is substantial evidence for the presence of an inward proton gradient in the mammalian small intestine and, therefore, it is likely that the H^+ gradient is the energy source for uphill transport of peptides [Ganapathy & Leibach, 1985]. It has been suggested that Na^+ plays an indirect role in peptide transport through the action of the Na^+-H^+ exchanger. This is located at the brush border membrane and secretes H^+ into the lumen, generating an inward proton gradient [Ganapathy & Leibach, 1985]. Together with the Na^+-K^+-ATPase at the basolateral membrane, the Na^+-H^+ exchanger generates and maintains the presence of the proton gradient, which drives di-/tripeptide transport [Ganapathy & Leibach, 1985].

FIGURE 1.4 *A model for intestinal transport depicting the role of Na,+ H+-gradient and cellular energy in the process (adapted from Ganapathy & Leibach, 1985)*



The direct involvement of H^+ gradient in di-/tripeptide transport has been investigated using Caco-2 cells loaded with the pH-sensitive fluorescent dye 2', 7',-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF), which monitors the intracellular pH of the cell [Thwaites *et al.*, 1993b]. All studies have shown a marked acidification of intracellular pH when perfused with dipeptides at the apical surface, which is consistent with H^+-flow into the cell [Thwaites *et al.*, 1993a, 1993b, 1993c, 1993g, 1994a]. Cytosolic acidification with apical application of Gly-Sar was observed at both apical pH 7.4 and pH 6.0 [Thwaites *et al.*, 1993a]. When the apical pH is 7.4, the pH gradient is minimal, but an acid microclimate (area of low pH adjacent to apical membrane) has been shown to exist *in vivo* [Lucus *et al.*, 1975] and *in vitro* [Purich *et al.*, 1973]. When the pH microclimate is not present the transapical electrical potential difference will be the driving force, indicating that peptide transport can occur in the neutral regions of the GI tract [Thwaites *et al.*, 1993a]. Demonstration of Gly-Sar net transport across Caco-2 cells in Na^+-free, pH-gradient conditions resulting in a transepithelial current flow, does not fit with the suggested hypothesis by Ganapathy & Leibach [Thwaites *et al.*, 1993g]. In order for transport to occur in Na^+-free conditions, a Na^+-independent mechanism which allows for coupling of apical H^+ influx to current flow at the basolateral membrane must exist. An H^+-K^+-ATPase which has been shown to contribute to intracellular pH regulation is expressed by Caco-2 cells [Abrahamse *et al.*, 1992]. This would allow coupling of H^+ efflux to cellular accumulation

of K^+, which would then diffuse *via* K^+ channels at the basolateral membrane, resulting in transepithelial current flow [Thwaites *et al.*, 1993g].

The involvement of protein kinase C in dipeptide transport has been investigated using Gly-Sar transport across Caco-2 cells [Brandsch *et al.*, 1994]. Treatment of the cells with phorbol-esters (phorbol 12-myristate 13-acetate {PMA}, phorbol 12, 13-dibutyrate {PDBu} and mezerein), which are activators of protein kinase C, resulted in a significant inhibition of Gly-Sar transport [Brandsch *et al.*, 1994]. 4a-Phorbol, 12, 13 didecanoate (PDD) and 4a-phorbol, which are not activators of protein kinase C had no effect on transport [Brandsch *et al.*, 1994]. Transport of L-Leu under the same conditions was not affected, indicating that the inhibition is not due to a non-specific effect [Brandsch *et al.*, 1994]. The inhibitory effect seen with activation of protein kinase C could be completely blocked by staurosporine (protein kinase C inhibitor). The inhibition of peptide transport was not prevented by co-treating with cycloheximide, an inhibitor of cellular protein synthesis, indicating that it is unlikely that the protein kinase C is acting by inhibiting synthesis of new transporter proteins [Brandsch *et al.*, 1994]. No change in intracellular pH was observed following treatment with the phorbol esters, ruling out the possibility that the inhibitory effect is a result of alteration in the transmembrane pH gradient. The catalytic function of the transport system appears to be inhibited by protein kinase C, possibly as a result of a change in the phosphorylation state of the transporter protein [Brandsch *et al.*, 1994].

1.7.4.2 CHEMICAL GROUPS ASSOCIATED WITH THE DTS

Cephalexin uptake into human placental BBMV was inactivated in the presence of the sulphydryl modifying reagents, *N*-ethylmaleimide and mercury (II) chloride ($HgCl_2$), suggesting that sulphydryl groups are essential for the DTS [Kudo *et al.*, 1989]. Cephradine accumulation and efflux in Caco-2, which has been shown to be inhibited by dipeptides, was also inhibited by the sulphydryl reagent *p*-chloromercuribenzene sulphonate, confirming that sulphydryl groups are associated with the DTS [Inui *et al.*, 1992].

Histidine groups have also been associated with dipeptide transport. Treatment of BBMV with diethylpyrocarbonate (DEPC), a histidine-modifying agent, has been shown to completely abolish the H^+-dependence of the oligopeptide transporter [Kato *et al.*, 1989: Kramer *et al.*, 1993].

1.7.4.3 BINDING PROTEIN OF THE DTS

Photoaffinity labelling has been used to isolate the protein involved in dipeptide uptake. A membrane glycoprotein of 127 kDa (kilo Dalton), which has been found to be directly involved in the uptake of small peptides and orally active β-lactam antibiotics, has been located in the brush-border membrane of enterocytes from pig, rabbit and rat [Kramer *et al.*, 1990b: Kramer *et al.*, 1995]. The presence of cefadroxil or cephalexin and dipeptides reduced the photo-affinity labelling of the glycoprotein when administered with

N-(4-azido[3,5-^{3}H]benzoyl)cephalexin and *N*-(4-azido[3,5-^{3}H]benzoyl)Gly-L-Pro [Kramer, 1987]. Sugars, amino acids and bile acids had no effect on the labelling process, illustrating that the 127 kDa protein is involved in the binding of cephalosporins and dipeptides [Kramer, 1987].

The above finding were confirmed in a study where membrane proteins from rabbit small intestinal BBMV were incorporated into liposomes using a gel filtration method [Kramer *et al.*, 1992]. Liposomes containing the 127 kDa binding protein were found to take up D-cephalexin stimulated by an inward H^+ gradient, which is consistent with di-/tripeptide/H^+ cotransport [Kramer *et al.*, 1992].

Using a monoclonal antibody (mAb) that blocked cephalexin uptake in Caco-2 cells, a protein band with an apparent mass of 120 ± 10 kDa has been detected in the membranes of Caco-2 cells [Dantzig *et al.*, 1994b]. Deglycosylation with endoglycosidase F before the membrane proteins were separated on a SDS-PAGE, shifted the immunoreactive band to approximately 100 kDa [Dantzig *et al.*, 1994b]. The 120 ± 10 kDa protein was found to be present in several human cell lines derived from the GI tract, but not in cell lines from other tissues (*e.g.* kidney) [Dantzig *et al.*, 1994b]. A complementary deoxyribonucleic acid (cDNA) library was prepared from 2-day post-confluent Caco-2 cells, and produced a clone of 3345 base pair, which encodes for a 832-amino acid putative transport protein of 92 kDa, which is consistent with the deglycosylated transporter [Dantzig *et al.*, 1994b]. The amino acid sequence of the cDNA of the cloned gene (*hpt*-1) revealed that the transporter protein shares several conserved structural elements with the cadherin superfamily of calcium-dependent, cell-cell adhesion proteins [Dantzig *et al.*, 1994b]. The *hpt*-1 gene encoding for the transporter protein was transfected into Chinese hamster ovary (CHO) cells, which showed two- to three-fold higher uptake of cephalexin over the transfectant control [Dantzig *et al.*, 1994b]. Cephalexin uptake was inhibited by excess unlabelled cephalexin, and also when the cells were incubated at pH 7.5, or pH clamped at pH 6.0, thus indicating the requirement of an inward H^+ gradient [Dantzig *et al.*, 1994b]. Similar results were achieved with Caco-2 cells under the same conditions, illustrating that the transporter properties were unchanged when expressed in CHO cells [Dantzig *et al.*, 1994b].

A protein of 130 kDa was expressed in the membrane of *Xenopus laevis* oocytes, after injection with mRNA derived from rat intestinal mucosal cells [Tamai *et al.*, 1994]]. This agrees with previous studies were a 127 kDa and 120 ± 10 kDa membrane protein have been isolated from rabbit small intestine [Kramer *et al.*, 1990b] and Caco-2 cells [Dantzig *et al.*, 1994b]. Ceftibuten, a tripeptide-like cephalosporin antibiotic, was found to be transported in the mRNA-injected oocytes [Tamai *et al.*, 1994]. Transport was found to be pH-dependent (H^+ gradient is the driving force), temperature-dependent and saturable, with dipeptides having an inhibitory effect, illustrating that ceftibuten is a substrate for the DTS [Tamai *et al.*, 1994].

1.7.4.4 CLONING OF THE DTS

Cloning and functional characterisation of the H^+-coupled oligopeptide transporter from rabbit small intestine has been carried out [Fei *et al.*, 1994]. The protein, consisting of a 707-amino-acid peptide transporter has been named PepT1. PepT1 mRNA has been found in the intestine, kidney, liver and in small amounts in the brain [Fei *et al.*, 1994]. PepT1 when expressed in *Xenopus laevis* oocytes, was shown to take up Gly-Sar 63-fold above that seen with the control oocytes [Fei *et al.*, 1994]. PepT1 was found to have a broad substrate specificity, with preference for peptides containing bulky aliphatic side chains [Fei *et al.*, 1994]. Uptake of peptides and cephalosporins *via* PepT1 evoked a large inward current, consistent with H^+-cotransport. PepT1 mediated uptake was found to be electrogenic, and independent of extracellular Na^+, K^+ and Cl^-, and of membrane potential [Fei *et al.*, 1994].

The cDNA encoding (hPEPT 1) for the H^+/peptide cotransporter in human intestine has recently been cloned using a probe derived form the rabbit H^+/peptide cotransporter cDNA [Liang *et al.*, 1995]. The encoded protein, is predicted to have a core molecular size of 78.9 kDa, and an isoelectric point of 8.6. The protein is predicted to consist of 708 amino acids, having 12 putative transmembrane domains with a long hydrophilic segment of approximately 200 amino acids, which contains seven sites for *N*-linked glycosylation, between domains 9 and 10 [Liang *et al.*, 1995]. Modelling of the protein revealed that the amino and carboxy termini would be located on the cytoplasmic side. Two possible sites for protein kinase C-dependent phosphorylation are also present [Liang *et al.*, 1995]. This agrees with a previous report that found the protein kinase C to be involved in the peptide/H^+ cotransporter system [Brandsch *et al.*, 1994]. A high degree of homology (81 % identity and 92 % similarity) exist between the amino acid sequence for this clone hPEPT 1, and that reported for the rabbit PepT1 clone [Fei *et al.*, 1994: Liang *et al.*, 1995]. However, very little homology to the H^+-coupled peptide transporter proteins in yeast and bacteria was observed. When the clone was expressed in *Xenopus laevis* oocytes and HeLa cells H^+ gradient-dependent peptide transport (Gly-Sar) was seen [Liang *et al.*, 1995]. The hPEPT 1 induced transport system appears to be specific for small peptides and peptidomimetic drugs, as di-/tripeptides and cephalosporins produced inhibition of transport, whereas amino acids did not [Liang *et al.*, 1995]. The induced transport system was found to be saturable, carrier-mediated and H^+-dependent [Liang *et al.*, 1995]. Surprisingly, the hPEPT 1 was found to have no significant similarity (16 % identity, 41 % similarity) with the *hpt*-1 protein isolated form Caco-2 cells [Dantzig *et al.*, 1994b]. The *hpt*-1 protein contains a single transmembrane domain whereas hPEPT 1 contains 12 transmembrane domains. Tissue distribution of the two proteins also differs [Liang *et al.*, 1995].

PepT2 is the latest member of the H^+/peptide cotransporter family has been cloned from the human kidney [Lui *et al.*, 1995]. It is distinct from hPEPT1, showing only 50 % identity and 70 % homology. PepT2 was not detectable in the small intestine [Lui *et al.*, 1995], and hPEPT1 was only found in the kidney in small amounts [Liang *et al.*, 1995].

The cDNA encoding for the rat peptide transporter (rat PepT1) has also been cloned in both the small intestine and kidney cortex [Saito *et al.*, 1995]. The rat PepT1 encodes the H^+-coupled di/tripeptide transporter in these tissues [Saito *et al.*, 1995]. The amino acid sequence of rat PepT1 has 77 % [Fei *et al.*, 1994] and 83 % [Liang *et al.*, 1995] identity with the rabbit PepT1 and human PepT1, respectively. The molecular mass of the rat PepT1 protein is 75 kDa, which is contradictory to the 127 kDa protein found in rat [Kramer, 1990], the 130 kDa protein expressed in oocyte membranes after injection of mRNA from rat small intestine [Tamai *et al.*, 1994] and the 120 kDa protein in Caco-2 cells [Dantzig *et al.*, 1994b]. The reason for the discrepancy in the molecular mass of the rat PepT1 protein compared to other isolated transporter proteins is not known. However, possible explanations are variable post-translational modifications, different protein migration rates during SDS-PAGE, and the existence of additional family members of the transporter proteins [Saito *et al.*, 1995].

1.7.4.5 LOCATION OF THE TRANSPORTER

The upper GI tract is usually considered as the primary site for absorption of drugs and small peptides ("window of absorption"). However, there is evidence indicating that the DTS may be expressed in the regions of the lower GI tract, such as the ileum and colon. The presence of the antigen (DTS transporter protein) has been located by immunohistochemical staining along the GI tract (jejunum, duodenum, ileum and colon) in normal human tissue [Dantzig *et al.*, 1994b]. The antigen was also expressed along the pancreatic ducts, suggesting a potential new site for the transport carrier [Dantzig *et al.*, 1994b]. The antigen was absent from specimens of the kidney, lung, liver, brain, adrenal gland and skin [Dantzig *et al.*, 1994b].

Gly-Gly and Gly-L-Leu absorption rates in the ileum and jejunum in man, have been shown to be similar or only slightly different. However, the intracellular hydrolysis rate is markedly greater in the ileum than in the jejunum [Adibi, 1971].

SQ-29852, which is a stable and specific probe for the DTS [Marino *et al.*, 1996: Nicklin *et al.*, 1996], has been shown to be absorbed from the various segments of the GI tract from rats (stomach, duodenum, jejunum, ileum and colon), with no significant difference in the absorption between the various sites [Marino *et al.*, 1996]. The DTS has been shown to be expressed in Caco-2 cell line, which is of colonic origination [Dantzig *et al.*, 1994b], supporting the above findings of DTS expression in the lower GI tract.

Several reports have indicated that the DTS is expressed to a lesser extent in the lower GI tract. Absorption of Gly-Gly, Gly-L-Leu and glycyl-L-alanine (Gly-L-Ala) in the human ileum has been shown to be lower (50 %) than that seen in the jejunum [Adibi, 1971: Silk *et al.*, 1974]. Similar results with absorption of L-Met-L-Met and Gly-L-Pro in rats have also been reported [Crampton *et al.*, 1973: Lane *et al.*, 1975].

Studies in rat jejunal and colonic everted rings found that the ACE-inhibitor, benazepril, was absorbed to a greater extent in the jejunum [Kim *et al.*, 1994]. Uptake in the

jejunal rings was found to be predominantly by a passive process with a small active carrier-mediated component, whereas uptake in the colonic rings was found to be exclusively *via* a passive process, suggesting that the DTS is not expressed in the colon [Kim *et al.*, 1994]. However, only 10 % of benazepril is absorbed *via* the DTS [Kim *et al.*, 1994], therefore it is not a specific probe, and it is unstable due to deesterification in the GI lumen or the enterocyte [Marino *et al.*, 1996].

1.7.4.6 MULTIPLE TRANSPORT SYSTEMS

The concept of more than one transport system responsible for the uptake and transport of di- and tripeptides in the intestine has been suggested for a number of years. However, most of the information has been gained from competition studies and so far only one piece of kinetic evidence has been provided.

The *in vivo* intestinal absorption of Gly-L-Pro and L-propylglycine (L-Pro-Gly) were investigated in rats by measuring the rates of disappearance of the dipeptides [Lane *et al.*, 1975] Excess (40 mM) L-Pro-Gly did not affect the rate of disappearance of 10 mM Gly-L-Pro. However, 40 mM Gly-L-Pro had a significant inhibitory effect on 10 mM L-Pro-Gly, suggesting that the two dipeptides may be absorbed *via* different carriers [Lane *et al.*, 1975]. This may also possibly be explained by different affinities of the two dipeptides for the carrier, with Gly-L-Pro showing a greater affinity than L-Pro-Gly, thus explaining the apparent lack of inhibition with L-Pro-Gly.

Competition studies using everted rat intestinal rings, indicated that Gly-L-Leu and Gly-L-Pro are absorbed *via* different transport systems [Cheeseman & Johnston, 1982]. It has been suggested that Gly-L-Leu and Gly-L-Pro share a transport system, but that there is an additional pathway for Gly-L-Leu absorption [Cheeseman & Johnston, 1982]. L-Leucyl-L-leucine (L-Leu-L-Leu) and L-leucyl-L-alanine (L-Leu-L-Ala) inhibited influx of Gly-L-Leu *via* the second route (not shared with Gly-L-Pro) in a non-competitive manner [Cheeseman & Johnston, 1982]. It has been suggested that the two L-Leu containing dipeptides, inhibit Gly-L-Leu uptake by an allosteric effect as a consequence of the two transport sites lying adjacent to each other [Cheeseman & Johnston, 1982].

Boyd and Ward suggested two possible transport systems after studying the electrical effects of dipeptide absorption. They proposed the presence of one transport system used by both carnosine and L-Leu-L-Leu and another only available to carnosine [Boyd & Ward, 1982]. Uptake studies of neutral dipeptides in hamster jejunum at pH 5, suggested two possible transporters [Matthews & Burston, 1984]. Competition studies revealed that Gly-Sar and Gly-Gly (dipeptides with the smallest side chains) were capable of completely inhibiting transport of each other in a competitive manner. However, they were weak inhibitors of dipeptides with bulkier side chains, *e.g.* L-alanyl-L-alanine (L-Ala-L-Ala), L-valyl-L-valine (L-Val-L-Val), and L-Leu-L-Leu [Matthews & Burston, 1984]. Gly-Sar was the exception to the rule and could inhibit transport of L-Val-L-Val. L-Ala-L-Ala, L-Val-L-Val, and L-Leu-L-Leu were all capable of completely inhibiting Gly-Sar transport in a

competitive manner [Matthews & Burston, 1984]. These results suggest that peptides with bulkier side-chains are possibly transported by a different transporter to peptides with smaller side-chains [Matthews & Burston, 1984].

The uptake of cephalexin, a cephalosporin which is reported to be a substrate for the DTS, was found to occur by similar carrier systems in Caco-2 and HT29 cells [Dantzig *et al.*, 1988, 1990]. However, the energy requirements of the transporters differed, indicating the possibility of more than one DTS [Dantzig *et al.*, 1988, 1990]. Transport of Gly-Gly across rat intestinal brush border membrane was inhibited by the dipeptides L-phenylalanylglycine (L-Phe-Gly) and carnosine, but not by ampicillin and cephradine, suggesting that the β-lactam antibiotics may utilise a different carrier system to that of the dipeptides [Iseki *et al.*, 1989].

A study using rabbit intestinal brush border membranes showed that cefixime, a cephalosporin, is actively transported by an H^+ gradient *via* the DTS only in the acidic region of the GI tract [Inui *et al.*, 1988]. Inhibition with dipeptides and aminocephalosporins was seen at pH 5 but not pH 7.5 [Inui *et al.*, 1988]. Cephradine was transported in both acidic and neutral pH regions, suggesting multiple transport systems for dipeptides [Inui *et al.*, 1988].

Kinetic analysis has revealed multiple carriers for dipeptides on the luminal membrane of renal proximal tubular cells in rabbit [Skopicki *et al.*, 1991]. The low affinity, high capacity carrier (K_m = 1.3 x 10^{-2} M, V_{max} = 4.6 x 10^{-8} mol mg^{-1} min^{-1}) and the high affinity low capacity carrier (K_m = 2.7 x 10^{-7} M, V_{max} = 7.8 x 10^{-13} mol mg^{-1} min^{-1}) also showed different inhibition profiles [Skopicki *et al.*, 1991]. Therefore, the possibility of a similar occurrence in the intestine is not an unrealistic one.

Uptake of SQ-29852 into Caco-2 cells was inhibited by the L-Ala-L-Pro, L-Phe-Gly, L-tyrosylglycine (L-Tyr-Gly) and L-tryptophylglycine (L-Trp-Gly), confirming that SQ-29852 is a substrate for the DTS [Nicklin *et al.*, 1996]. However, the cephalosporins, cephalexin and cephradine, which are widely used as probes for the DTS due to their stability, had no significant effect on SQ-29852 uptake [Nicklin *et al.*, 1996]. This suggests the possibility of different transporters in Caco-2 cells, a cephalosporin sensitive and insensitive pathway. However, the lack of inhibition with the cephalosporins may be due to a much lower affinity for the carrier than SQ-29852.

The presence of at least three transport systems for dipeptides and peptidomimetic drugs has recently been proposed [Muranushi *et al.*, 1995]. S-1090 (a new oral cephem) uptake in rat intestinal BBMV has produced some interesting inhibition profiles, which suggest that the heterogeneity may depend on the structure of the *N*-terminal amino acid [Muranushi *et al.*, 1995]. The first transporter is for aliphatic (relatively hydrophilic) peptides, such as Gly-Gly and L-glutamyl-L-alanyl-L-alanine (L-Glu-L-Ala-L-Ala). The second is for aromatic (hydrophobic) peptides, such as L-phenylalanyl-L-alanyl-L-alanine (L-Phe-L-Ala-L-Ala), and the third is for peptides with a heterocyclic amino acid (*e.g.* L-histidine {L-His} or L-tryptophan {L-Trp}) [Muranushi *et al.*, 1995]. Ceftibuten is a

substrate for the first transporter, while cefaclor is a substrate for the second transporter, but partly recognised by the first transport system [Muranushi *et al.*, 1995]. S-1090 is proposed to be transported by the third system that recognises peptides with L-His in the *N*-terminal position [Muranushi *et al.*, 1995]. However, amino acids had inhibitory effects on S-1090 uptake, therefore S-1090 appears to be a non-specific probe for the DTS and the inhibition profiles may be a result of interaction with amino acid transporters.

The possibility that the apical and basolateral transporters differ has also been suggested. Basolateral Gly-Sar and cephalexin were both found to cause cytosolic acidification in Caco-2 cells, with acidic basolateral solutions (pH 6.0), providing direct evidence for a H^+-coupled transporter at the basolateral membrane [Thwaites *et al.*, 1993a] Cephalexin was able to inhibit basal-apical Gly-Sar transport. When the basolateral solution was at pH 7.4, only cephalexin was able to induce cytosolic acidification, indicating that although a common carrier for Gly-Sar and cephalexin may exist at the basolateral membrane, cephalexin transport involves distinct features, which may involve multiple transporter systems [Thwaites *et al.*, 1993a].

The suggestion that the apical and basolateral DTS are different is highlighted by bestatin uptake into Caco-2 cells [Saito & Inui, 1993]. Absorption was found to be accumulated in the cells and transported unidirectionally to the basolateral surface [Saito & Inui, 1993]. Uptake at the apical surface was pH-dependent (maximum at pH 6.0), whereas uptake at the basolateral membrane was pH-independent [Saito & Inui, 1993]. The transporters at the apical and basolateral surface also differed with regard to kinetic parameters and inhibition profiles [Saito & Inui, 1993]. The basolateral transporter was found to be more sensitive to the sulphydryl agent, p-chloromercribenzene (PCNBS) than the apical, indicating that sulphydryl groups are an essential component of both transporters [Saito & Inui, 1993]. These findings indicate that the DTS exist on both the apical and basolateral membranes of Caco-2 cells, but the basolateral transporter is distinct from that of the apical H^+-peptide/ cotransporter [Saito & Inui, 1993].

Further studies are required in order to clarify the possibility of multiple transport systems for dipeptides. Differing affinities of compounds for the DTS may explain the inhibition profiles to date and, therefore, evidence from kinetic parameters and cloning of a second transporter would provide direct conformation.

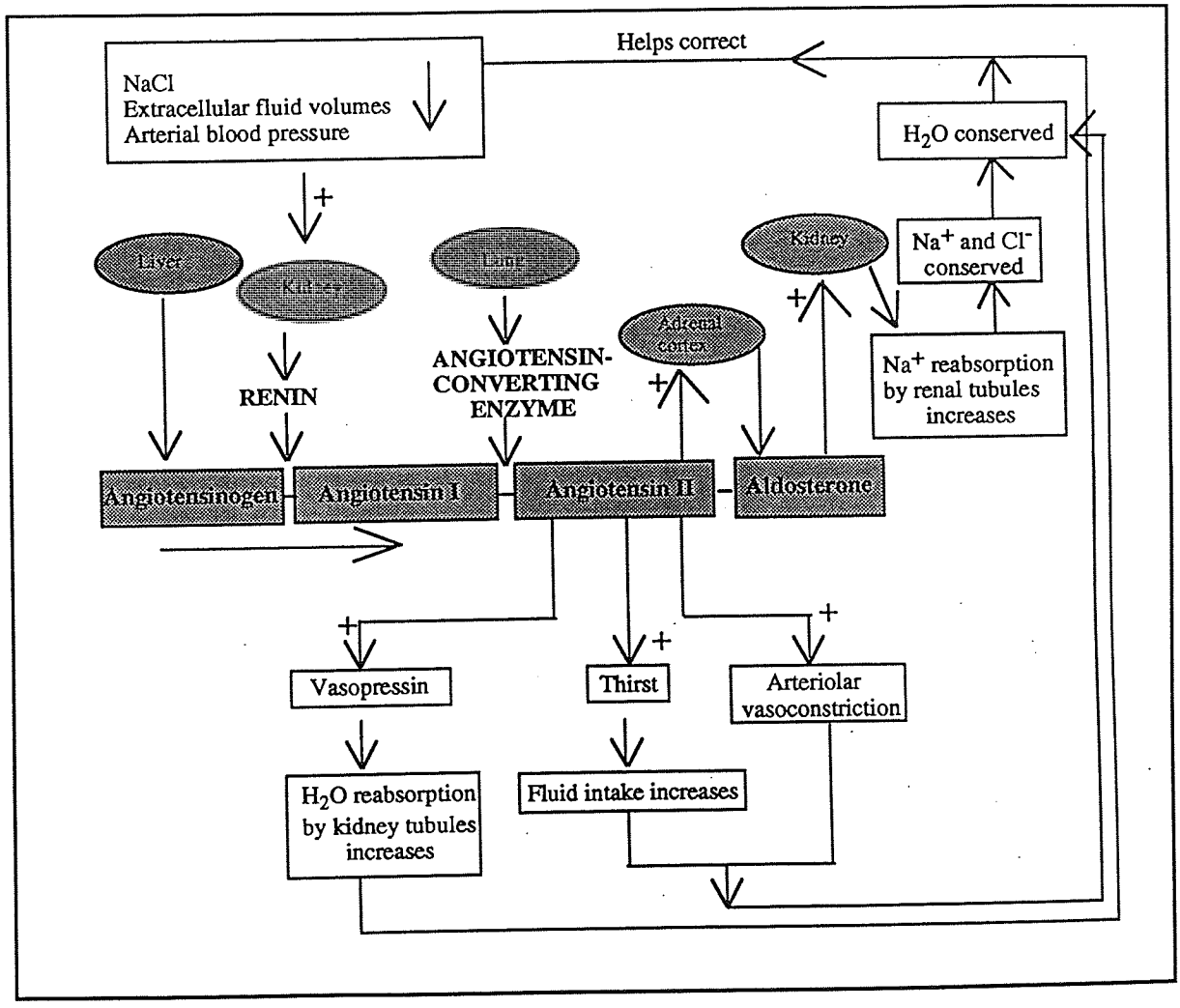
1.8 PEPTIDOMIMETIC DRUGS

Peptidomimetic drugs are those which have structural similarities to peptides. ACE-inhibitors, β-lactam antibiotics, renin and thrombin inhibitors are all peptidomimetic drugs which have been reported to have a degree of active transport *via* the DTS. Transport mechanisms of thrombin inhibitors will be discussed in section 1.10.5.

1.8.1 ACE-INHIBITORS

ACE (Kinase II) is a liver-produced protein that is found as either a membrane-bound or a circulating molecule in the body fluids. It is a glycoprotein consisting of a single subunit which has a transmembranous orientation. It is associated with the membrane *via* a hydrophobic segment and possesses a cytoplasmic tail [Naim, 1992]. It converts the inactive angiotensin I (Ag I) to angiotensin II (Ag II), which is a highly active octapeptide II, by the removal of two or more amino acids. Ag II is a potent vasoconstrictor and stimulator of aldosterone secretion, which plays an important role in the Renin-Angiotensin-System (RAS) (Figure 1.5) controlling blood pressure.

FIGURE 1.5 *RAS (adapted from Sherwwod, L., 1993)*



ACE-inhibitors are a group of synthetically (with the exception of teprotide) produced peptide analogues, which have the clinical effect of lowering the blood pressure by reducing peripheral vascular resistance and aldosterone-mediated Na^+-reabsorption. They act by inhibiting ACE Kinase II and thus preventing the production of Ag II. They have been extensively used in the treatment of hypertension and congestive heart failure due to reducing the afterload.

The first and only natural ACE-inhibitor, teprotide (a nonapeptide) was isolated from the venom of *Bothrops jararaca*, a Brazilian pit viper. It is an effective ACE-inhibitor but clinical testing was limited due to its scarcity, the need for parenteral administration and the development of captopril [DiBianco, 1986].

Captopril was the first orally active ACE-inhibitor. Its antihypertensive action, when used as a sole treatment, matches that of the β-antagonists [Ball, 1985]. Captopril is absorbed rapidly from the GI tract in normal individuals with a plasma appearance within 15 minutes following administration. It has a good oral bioavailability of between 60-75 % [Duchin *et al.*, 1982]. Peak plasma concentrations are reached after 1 h, but the duration of action on the RAS in peripheral blood is short, due to being extensively metabolised to disulphide dimers. However, twice daily administration usually controls the blood pressure over 24 h period [Ball, 1985].

Captopril absorption was originally proposed to be *via* a passive mechanism, as absorption was dose- and concentration-independent [Duchin *et al.*, 1982]. However, one piece of evidence that does not support this is the fact that absorption of captopril is reduced 35 % in the presence of food, suggesting that dietary protein products are competing with the drug for transport [Singhvi *et al.*, 1982: Williams & Sugerman, 1982]. Captopril may react with proteins [Wong *et al.*, 1981] in the lumen through mixed disulphide formations and, therefore, restrict transport, rather than competing for it. A study using a single-pass perfusion method in fasted rats showed that captopril is very permeable in the small intestine but not in the colon. The intestinal permeability of the drug was found to be concentration-, pH-, energy- and Na^+-dependent. Competition studies with the dipeptides Gly-Gly and Gly-L-Pro and cephradine showed a significant inhibition in permeability, indicating an active transport system [Hu & Amidon, 1988]. Harmaline and the metabolic inhibitor DNP caused a significant reduction in captopril transport across rat intestine, indicating the involvement of a Na^+- and energy-dependent process [Zhou & Li Wan Po, 1994]. The evidence suggests that captopril transport is mediated *via* both a passive and active transport mechanism, utilising the DTS.

Enalapril is an L-Ala-L-Pro derivative containing two amino groups, one in the proline ring and the second in an α-position to the peptide bond. It is an ethyl ester of the active drug enalaprilat, which produces well-defined clinical effects due to binding tightly to ACE, being more potent than captopril [Gross *et al.*, 1981]. Enalapril is converted to the active drug *via* deesterification *via* hepatic biotransformation [Friedman & Amidon, 1989b]. It has a good bioavailability, with 60-70 % of the oral dose being absorbed [Kudo & Cody 1985]. Maximum plasma concentrations are seen 1 h after administration and rapid clearance by de-esterification in the liver to enalaprilat is seen within 4 h. It has a longer duration of action than captopril and therefore single daily dosing is sufficient [Kudo & Cody, 1985: Ball, 1985]. Enalapril has better absorption than the active drug enalaprilat, which is < 10 % absorbed in man [Kudo & Cody, 1985]. This is probably due to a higher

distribution coefficient which increases passive diffusion, < 0.001 compared to 0.07 at pH 7, for enalaprilat and enalapril, respectively [Ranadive *et al.*, 1992].

A single pass perfusion study in rats found that enalapril is well absorbed from the jejunum, with a saturable uptake component (K_m = 0.07 mM) [Friedman & Amidon, 1989b]. Absorption was found to be concentration-dependent, and inhibited in the presence of L-Tyr-Gly, but not by cephradine and amino acids, suggesting an active transport system *via* the DTS [Friedman & Amidon, 1989b]. Enalapril has been shown to inhibit uptake of cephradine in both rat and rabbit BBMV [Yuasa *et al.*, 1993]. Further studies revealed that enalapril, inhibited the uptake of cephradine into rabbit BBMV by a non-competitive mechanism with a K_i of 2.6 mM when on the *cis* side (outside) of the vesicles [Yuasa *et al.*, 1994]. However, enalaprilat inhibited cephradine uptake in a competitive manner with a K_i of 5.4 mM [Yuasa *et al.*, 1994]. The presence of an enalapril-specific inhibitory-binding site on the peptide carrier has been suggested [Yuasa *et al.*, 1994]. Enalapril, when presented at the *trans* side (inside) of the vesicles, produced inhibition of cephradine uptake, indicating a possible reduction in carrier availability due to a trapping mechanism [Yuasa *et al.*, 1994].

Using an *in vivo* rat intestine system, enalapril transport was found to be an active process with the following kinetic parameters; $K_t = 0.81 \pm 0.23$ mM, $J_{max} = 0.58 \pm 0.37$ μmol h^{-1} cm^{-2}, with a passive permeability constant of 0.56 ± 0.04 cm h^{-1} [Swaan *et al.*, 1995]. Enalaprilat transport was found to be *via* a passive diffusional process, having a permeability constant of 0.51 ± 0.04 cm h^{-1}, agreeing with the low oral absorption recorded by Kudo & Cody [Kudo *et al.*, 1985: Swaan *et al.*, 1995]. Enalapril and enalaprilat were both found to inhibit transport of cephalexin, with K_i's of 0.15 mM and 0.28 mM, respectively [Swaan *et al.*, 1995]. However, a recent study using everted rat intestinal rings reported no inhibition of enalapril uptake with seven β-lactam antibiotics [Morrison *et al.*, 1996]. Enalapril uptake was also found to be independent of concentration in the incubation bath, suggesting a passive mechanism [Morrison *et.al.*, 1996]. The absorption of enalapril, when given orally to rats and dogs over a wide concentration range (1-50 mg kg^{-1} for rats, and 0.06-6 mg kg^{-1} for dogs) was found to be independent of dose, again indicating a passive process [Morrison *et al.*, 1996]. The inconsistency in the results, and the fact that enalapril was capable of inhibiting cephalexin transport [Swaan *et al.*, 1995] yet the β-lactam antibiotics failed to inhibit enalapril absorption [Morrison *et al.*, 1996], may indicate that enalapril and enalaprilat are not substrates for transport *via* the DTS, but may bind to the transporter and therefore blocking the receptor site. This would also explain the non-competitive inhibition by enalapril seen in rabbit BBMV [Yuasa *et al.*, 1994].

Lisinopril is a nonsulphydril L-lysyl-L-proline (L-Lys-L-Pro) analogue. Its oral bioavailability is only 25-29 %, but it has a longer duration of action than captopril [Friedman & Amidon, 1989a]. Perfusion studies using rat jejunum found that the permeability of lisinopril was concentration-dependent and decreased when coperfused with the dipeptide L-Tyr-Gly and the cephalosporin, cephradine [Friedman & Amidon, 1989a]. These results suggest that lisinopril is absorbed by an active mechanisms *via* the DTS

[Friedman & Amidon, 1989a]. Passive absorption of lisinopril was found to be negligible, which can be attributed to it being highly charged at jejunal pH, due to being a diacid [Friedman & Amidon, 1989a]. The active transport mechanism of lisinopril absorption was confirmed in a recent *in vivo* study, where lisinopril was also shown to inhibit the transport of cephalexin across rat intestine, having a K_i of 0.39 mM [Swaan *et al.*, 1995]. Lisinopril showed a lower affinity for the carrier when compared to enalapril and enalaprilat [Swaan *et al.*, 1995]. Molecular modelling revealed that the decreased affinity for the carrier was due to intramolecular hydrogen bond formation [Swaan *et al.*, 1995].

Benazepril is an L-phenylalanyl-L-alanyl-L-proline (L-Phe-L-Ala-L-Pro) like ester prodrug of the active drug benazeprilat, which has been shown to have an oral bioavailability of 37 % in humans [Waldmeier & Schmid, 1989]. Benazepril has been reported to undergo carrier-mediated transport *in situ* [Yee & Amidon, 1990]. Absorption in the jejunum and colon of dogs and also in rat everted intestinal rings were assessed [Kim *et al.*, 1994]. Uptake was found to be 3.5 times higher in the jejunal rings compared to those from the colon [Kim *et al.*, 1994]. A weak concentration-dependence was seen in the jejunal rings, with only 10 % of uptake being attributed to a carrier-mediated process at a 1 mM concentration [Kim *et al.*, 1994]. Di- and tripeptides caused inhibition of jejunal uptake whereas amino acids had no effect, indicating that the carrier-mediated uptake is *via* the DTS [Kim *et al.*, 1994]. No inhibition was observed for colonic uptake indicating an exclusively passive process [Kim *et al.*, 1994]. The *in vivo* (dog) studies produced the same results with jejunal administration resulting in higher (5.5 times) plasma levels than colonic administration [Kim *et al.*, 1994].

Fosinopril is an α phosphinic L-Ala-L-Pro type ACE-inhibitor [Friedman & Amidon, 1989b]. It is a prodrug which is converted to its active diacid drug, fosinoprilat, *via* deesterification in the GI mucosa and liver [Duchin, 1991]. Intestinal absorption was investigated using a single-pass perfusion method in rats [Friedman & Amidon, 1989b]. Fosinopril was well absorbed over a wide range of concentrations [Friedman & Amidon, 1989b]. No evidence for an active uptake mechanism was present, as L-Tyr-Gly and cephradine had no effect on transport [Friedman & Amidon, 1989b].

TABLE 1.2 *ACE-inhibitor interaction with the DTS*

ACE-inhibitor	DTS	Evidence
Benazepril	+	Carrier-mediated transport in situ [Yee & Amidon, 1990].
	+	Uptake showed weak concentration-dependence in jejunal rings of rats, and uptake was *via a* carrier-mediated process, which was inhibited by di- and tripeptides [Kim *et al.*, 1994].
Captopril	-	Absorption, dose- and concentration-independent [Duchin *et al.*, 1982]
	+	Absorption is decreased 35 % with food [Singhvi *et al.*, 1982: Williams & Sugerman, 1982]
	+	Inhibited by Gly-Gly, Gly-L-Pro and cephradine [Hu & Amidon, 1988].
Enalapril	+	Absorption in rat jejunum was *via* an active process, concentration-dependent, inhibited by L-Tyr-Gly [Friedman & Amidon, 1989b].
	+	Enalapril inhibits cephradine uptake in rat and rabbit BBMV. Non-competitive inhibition in rabbit BBMV [Yuasa *et al.*, 1994].
	+	Enalapril transport *via* an active process in rat intestine, and inhibits cephalexin transport [Swaan *et al.*, 1995].
	-	Seven β-lactams failed to inhibit enalapril uptake into rat intestinal rings, enalapril uptake was independent of concentration. [Morrison *et al.*, 1996].
	-	Absorption in rats and dogs was dose-independent [Morrison *et al.*, 1996].
Enalaprilat	+	Enalaprilat inhibited cephradine uptake into rabbit BBMV in a competitive manner [Yuasa *et al.*, 1994].
	+ / -	Enalaprilat transport in rat intestine *via* a passive process, however, inhibited cephalexin transport [Swaan *et al.*, 1995].
Fosinopril	-	L-Tyr-Gly and cephradine failed to inhibit transport, no evidence for active uptake mechanism [Friedman & Amdion, 1989b].
Lisinopril	+	Permeability in rat jejunum was concentration-dependent and inhibited by L-Tyr-Gly and cephradine [Friedman &, 1989a].
	+	Inhibited cephalexin transport across rat intestine [Swaan *et al.*, 1995].

\+ Evidence for interaction with the DTS,

\- No evidence for interaction with the DTS.

1.8.2 β-LACTAM ANTIBIOTICS

β-Lactam antibiotics are regarded as peptide mimetics of the dipeptide D-alanyl-D-alanine (D-Ala-D-Ala) [Catnach & Fairclough, 1994], and can be broadly classed into two groups, the penicillins and the cephalosporins. The penicillins have the β-lactam fused to a thiazolidine ring forming a 6-β-acylaminopenicillanic acid structure [Bergan, 1984]. The cephalosporins have a 3-cephem structure which is formed from the dihydrothiazine ring and the β-lactam [Bergan, 1984]. β-Lactam antibiotics are usually weak acids, with low lipophilicity [Catnach & Fairclough, 1994]. They are predicted to be not easily transported across the apical membrane of enterocytes, as the pH partition theory indicates that they would be completely ionised at physiological pH [Catnach & Fairclough, 1994]. However, the aminopenicillins (amoxicillin and cyclocillin) and the aminocephalosporins (cefaclor, cephalexin and cephadroxil) are almost completely absorbed from the gut [Kwan & Rogers, 1983]. Information regarding the absorption mechanisms of the β-lactams is somewhat contradictory and will be further discussed. A possible explanation for the discrepancies between studies, are the use of different experimental systems and the fact that the antibiotics that are rapidly absorbed have saturable kinetics which are easily identified and those associated with slow absorption are more difficult to define. There is also evidence to suggest that β-lactam antibiotics are absorbed by more than one carrier system.

In vitro uptake of cyclacillin and cephalexin in rat everted jejunum was found to involve an active transport mechanism [Nakashima *et al.*, 1984b]. Several dipeptides were found to inhibit uptake in a competitive manner. Furthermore, Gly-Gly uptake was inhibited by cyclacillin, cefadroxil, cephalexin and cephradine, suggesting a shared mechanism for uptake of dipeptides and antibiotics [Nakashima *et al.*, 1984b]. An *in situ* experiment using rat intestines found that the α-aminocephalosporins (cefadroxil and cephalexin) and the α-aminopenicillin cyclacillin were absorbed *via* a saturable process, that was inhibitable by dipeptides, confirming the involvement of the DTS [Nakashima *et al.*, 1984a]. The aminocephalosporin, cephradine was found to be taken up into rat intestinal BBMV by a saturable, active carrier-mediated process, with a K_m of 9.4 mM [Okano *et al.*, 1986b]. Uptake was shown to be Na^+-independent, stimulated in the presence of an inward H^+ gradient, and inhibited in the presence of dipeptides but not amino acids, suggesting uptake is *via* the DTS [Okano *et al.*, 1986b]. Cephradine uptake into rabbit intestinal BBMV was found to be stimulated by the counter-transport of dipeptides, providing evidence for the presence of a common carrier system [Okano *et al.*, 1986a]. An overshoot phenomenon was seen when an inward H^+ gradient stimulated uptake against a concentration gradient [Okano *et al.*, 1986a]. The uptake of cefadroxil, cefaclor and cephalexin were all stimulated in the presence of an H^+ gradient, whereas uptake of cefazolin and cefotiam, which lack an α-amino group, were not affected by the presence or absence of an H^+ gradient [Okano *et al.*, 1986a]. This provides direct evidence of the involvement of an inward H^+ gradient and the transfer of a positive charge in the absorption of aminocephalosporins possessing an α-amino group. However, the finding that uptake of cefazolin and cefotiam did not involve an

H^+ gradient, and therefore did not involve the DTS, is not surprising considering that these cephalosporins are known to be poorly absorbed across the intestine, and are therefore administered parenterally [Miyazaki *et al.*, 1982]. The initial idea that only β-lactams possessing an α-amino group were substrates for the transporter was questioned when the aminothiazolyl cephalosporins cefixime and ceftibuten, were found to be well absorbed from the gut [Catnach & Fairclough, 1994]. Transport of cefixime and the *cis* isomer of ceftibuten, which both lack the α-amino group were found to be driven by an inward H^+-gradient [Inui *et al.*, 1988: Yoshikawa *et al.*, 1989].

A model has been proposed for the molecular mechanism of the H^+-dependent DTS [Kramer *et al.*, 1995]. This has been based on the fact that uptake of cephalosporins lacking the α-amino group was not stimulated by a H^+-gradient [Okano et al., 1986a], and that uptake of cephalosporins lacking this structure are not affected by the histidine-modifying agent DEPC [Kramer *et al.*, 1988]. DEPC inactivation of the transporter could be inhibited by β-lactam antibiotics and oligopeptides with a free α-amino group [Kramer *et al.*, 1995]. However, cephalothin or cefotiam, which lack the α-amino group, are strong inhibitors of cephalexin uptake and photoaffinity labelling of the 127 kDa transport protein, but were unable to prevent deactivation with DEPC [Kramer *et al.*, 1995]. A proton donor-acceptor relationship has been proposed to exist between the α-amino group of the substrate and the histidine residue of the transporter protein, which leads to the translocation of the substrate across the brush border membrane [Kramer *et al.*, 1995]. However, cefixime, which is dianionic, was also able to protect the transporter form DEPC inactivation, suggesting that the carboxymethoxyimino function at position 7 of the cephem nucleus is capable of interacting with the histidine residues [Kramer *et al.*, 1995].

Cephalexin is absorbed *via* the DTS in rat intestine [Tamai *et al.*, 1988]. D-Cephalexin was found to be absorbed *via* a saturable process that was competitively inhibited by L-cephalexin, with a K_i of 0.67 ± 0.09 mM [Tamai *et al.*, 1988]. No appreciable uptake of L-cephalexin was observed, however, the breakdown product of L-cephalexin was detected in the serum after oral administration, indicating that L-cephalexin is absorbed, but not detectable due to hydrolysis [Tamai *et al.*, 1988]. L-Cephalexin was found to have a higher affinity for the transporter demonstrating stereospecific transport of cephalosporins *via* the DTS [Tamai *et al.*, 1988].

The five cephalosporin antibiotics, cefaclor, cefadroxil, cefatrizine, cephalexin and cephradine have all been found to have significant carrier-mediated absorption in an *in situ* model using rat jejunum [Sinko & Amidon, 1988]. Absorption of cefatrizine and cephradine were shown to include a small passive component [Sinko & Amidon, 1988]. Cefaclor permeability in the colon was low and found to be *via* a passive process [Sinko & Amidon, 1988]. Cephalexin was able to competitively inhibit cefadroxil permeability in rat small intestine [Sinko & Amidon, 1989]. A decrease in β-lactam permeability was also seen when coperfused with di- and tripeptides, confirming that β-lactam absorption is *via* the DTS in rat small intestine [Sinko & Amidon, 1989].

Transport characteristics of β-lactam antibiotics and dipeptides were compared across rat intestinal brush border membranes [Iseki *et al.*, 1989]. The uptake rate of Gly-Gly and cephradine was stimulated in the presence of an H^+ gradient, whereas that of ampicillin was not [Iseki *et al.*, 1989]. Stimulation of cephradine with the same H^+ gradient, was lower than that of the dipeptide, but cephradine uptake was greater than that of Gly-Gly [Iseki *et al.*, 1989]. L-Phe and carnosine inhibited the uptake of Gly-Gly, whereas cephradine and ampicillin had no effect [Iseki *et al.*, 1989]. The H^+ gradient appears to have differing effects on uptake of the three substances tested, and suggests that cephradine and ampicillin are taken up *via* a different mechanism for that of Gly-Gly.

Uptake of the aminopenicillins (ampicillin and amoxicillin) and the aminocephalosporins (cephalexin, cephradine and cefadroxil) and cefazolin were compared to that of the passively absorbed compound, L-glucose, in the rat intestinal brush border membrane [Sugawara *et al.*, 1990]. The time course and degree of uptake of the mentioned drugs were found to be similar to that seen with L-glucose, with no overshoot phenomenon being observed for any of the compounds tested in the presence of an inward H^+ gradient [Sugawara *et al.*, 1990]. These results suggest that the β-lactam antibiotics tested permeate the membrane mainly by a passive mechanism [Sugawara *et al.*, 1990]. However, this does not explain the difference in absorption seen with the drugs, suggesting the involvement of another process. Sugawara *et al.* have subsequently shown that ceftibuten and its analogues exhibited an overshoot phenomenon in the presence of an H^+ gradient against a concentration gradient, contradicting their previous study [Sugawara *et al.*, 1991a, b].

Photoactive derivatives of β-lactam antibiotics and dipeptides were used to photoaffinity label a binding protein of molecular weight 127 kDa from the rabbit small intestinal brush border membrane [Kramer *et al.*, 1990b]. Treatment of BBMV with an antiserum raised against this protein was found to significantly inhibit the efflux of cephalexin from the vesicles, suggesting that the 127 kDa protein is involved in the transport process [Kramer *et al.*, 1990b]. The protein was later reconstituted into liposomes, which were found to take up D-cephalexin in the presence of an inward H^+ gradient [Kramer *et al.*, 1992]. Photoaffinity labelling with benzylpenicillin revealed that the binding specificity of the protein incorporated into the liposomes was identical to that of the original BBMV [Kramer *et al.*, 1992]. The uptake system was found to be stereospecific for D-cephalexin, with no significant uptake of the L-isomer, however, both enantiomers bound to the 127 kDa protein [Kramer *et al.*, 1992]. As a result of the above findings, Kramer *et al.* suggested that the 127 kDa protein was the intestinal peptide transporter or a component of it [Kramer *et al.*, 1992].

Ceftibuten, which lacks the α-amino group at position 7 of the cephem skeleton was taken up into human jejunal BBMV by a process that was stimulated in the presence of an H^+ gradient, showing an overshoot phenomenon, whereas no effect was seen with cefixime, cefaclor and cephalexin. This suggests that these antibiotics are absorbed *via* different

carrier systems, an H^+ gradient dependent and an independent process [Muranushi *et al.*, 1989: Sugawara *et al.*, 1991b].

The uptake of several aminopenicillins was investigated in an *in situ* rat intestinal model [Oh *et al.*, 1992]. All of the compounds tested produced saturable absorption kinetics indicating a carrier-mediated process [Oh *et al.*, 1992]. Inhibition studies supported this, with cephradine producing competitive inhibition of amoxicillin [Oh *et al.*, 1992]. Amoxicillin with a K_m of 0.058 ± 0.026 mM demonstrated the highest affinity for the carrier, whilst ampicillin and cyclacillin displayed much lower affinities for the carrier with K_m values of 15.80 ± 2.9 mM and 14.00 ± 3.30 mM, respectively [Oh *et al.*, 1992].

Cephalosporin transport across intestinal BBMV in man, rat and rabbit has highlighted the occurrence of interspecies differences with regards to transport [Sugawara *et al.*, 1992]. Ceftibuten was taken up into BBMV of man and rat against a concentration gradient (overshoot phenomenon) in the presence of an inward H^+ gradient, whereas cephradine was not [Sugawara *et al.*, 1992]. However, in rabbit BBMV, an overshoot phenomenon in the presence of an inward H^+ gradient was seen with both cephalosporins [Sugawara *et al.*, 1992]. Ceftibuten uptake was inhibited by a ceftibuten analogue, whereas cephradine uptake was not inhibited by any of the cephalosporins tested, in human and rat BBMV [Sugawara *et al.*, 1992]. However, both ceftibuten and cephradine uptake in rabbit BBMV was inhibited by cephalosporins and dipeptides [Sugawara *et al.*, 1992]. These results highlight the differences between species and illustrate the unsuitability of the rabbit model for investigating transport of β-lactam antibiotics. Ceftibuten uptake in human and rat BBMV was found to be partially or non-competitively inhibited by the dipeptide L-Ala-L-Pro, indicating that transport is *via* a different carrier to that for L-Ala-L-Pro [Sugawara *et al.*, 1994].

Cephradine has been shown to be taken up into rabbit intestinal cells by a concentrative mechanism which was inhibitable by Gly-Sar [Tomita *et al.*, 1995]. Uptake was found to involve the H^+/dipeptide cotransporter and the Na^+/H^+ exchanger [Tomita *et al.*, 1995]. Uptake was inhibited by a range of di-, tripeptides and β-lactam antibiotics in both rat and rabbit BBMV, indicating transport is *via* the peptide transporter [Yuasa *et al.*, 1993]. Cephradine uptake into rabbit BBMV was found to be non competitively inhibited by the ACE-inhibitor enalapril, which has been explained due to the presence of a specific inhibitory binding site on the peptide carrier [Yuasa *et al.*, 1994]. However, as previously mentioned, the rabbit is not a representative model for the human situation, and therefore further studies are required using a more appropriate model.

A more recent study by Kramer *et al.* has shown that the H^+-dependent uptake of cephalexin into rabbit intestinal BBMV was inhibited to some degree depending on their chemical structure, by all 30 β-lactam antibiotics tested, regardless of whether they are orally absorbed or not [Kramer *et al.*, 1995]. All the β-lactam antibiotics tested also decreased the photoaffinity labelling of the 127 kDa binding protein, illustrating a direct interaction with the DTS [Kramer *et al.*, 1995].

S-1090 which is a new oral cephem, does not have an α-amino or a carboxyl group in the side chain at the 7-position of the cephem skeleton, but has a hydroxyimide instead [Muranushi *et al.*, 1995]. S-1090 uptake in rat intestinal BBMV was stimulated by an H^+ gradient, however overshooting was not observed [Muranushi *et al.*, 1995]. Although the di- and tripeptides with aliphatic side-chains (L-Ala and Gly amino acid components) did not inhibit uptake, those with L-His, L-Pro or L-Try in the *N*-terminal position did [Muranushi *et al.*, 1995]. L-Histidyl-L-alanine (L-His-L-Ala) and L-histidylglycine (L-His-Gly) were found not only to have an inhibitory effect, but also a countertransport effect when the vesicles were preloaded with the dipeptides. The cephalosporins cefaclor and cephalexin were without an inhibitory effect, whereas ceftibuten showed a marked inhibition [Muranushi *et al.*, 1995]. Surprisingly, amino acids also had an inhibitory effect , which was suggested to be a result of binding to the transporter protein and not competition for transport [Muranushi *et al.*, 1995]. The dipeptide L-Ala-L-Ala and the tripeptide L-Glu-L-Ala-L-Ala which have been shown to strongly inhibit cephalexin [Sugawara *et al.*, 1992] and ceftibuten [Muranushi *et al.*, 1989] uptake, failed to inhibit S-1090 [Muranushi *et al.*, 1995]. This may suggest that S-1090 was taken up *via* a different carrier to that for cephalexin, cefaclor and ceftibuten [Muranushi *et al.*, 1995].

Further information to suggest the existence of more than one transport system for β-lactam antibiotics has been provided. Cephalexin uptake into rabbit intestinal BBMV and photoaffinity labelling of the 127 kDa transporter protein was competitively inhibited by benzylpenicillin, dipeptides, α-amino-β-lactam antibiotics like cefadroxil, and the dianionic cephalosporin cephalexin [Kramer *et al.*, 1995]. These results show that a molecular interaction with the transporter is occurring with all the substrates. Heat treatment impaired uptake of α-amino-β-lactam antibiotics [Kramer et *al.*, 1995], whereas it had no effect on uptake of cefixime [Kramer *et al.*, 1992: Kramer *et al.*, 1993]. The anion transport inhibitor 4, 4 -diisothiocyanatostilbene-2, 2 -disulphonic acid (DIDS), inhibited cefixime uptake but had no effect on that of cephalexin [Inui *et al.*, 1988], thus suggesting that the transport of dianionic cephalosporins, although having interaction with the DTS, are transported by a second carrier.

Penicillin-G uptake in rabbit intestinal BBMV was found to be a saturable carrier-mediated process, showing H^+-dependency with maximum uptake being seen at pH 4.5 [Poschet *et al.*, 1996]. Uptake was inhibited by ampicillin, penicillin-V, cefadroxil, cephalexin, cephalothin, cephradine, L-carnosine, Gly-L-Ala, glycyl-L-tyrosine (Gly-L-Tyr), and glycylglycylglycine (Gly-Gly-Gly), indicating penicillin-G is a substrate for the DTS [Poschet *et al.*, 1996]. Gly-Sar stimulated uptake by 92 % [Poschet *et al.*, 1996]. Counter transport studies showed that BBMV loaded with Gly-Sar, inhibited penicillin-G uptake, suggesting that an interaction at the same site on the transporter is taking place [Poschet *et al.*, 1996].

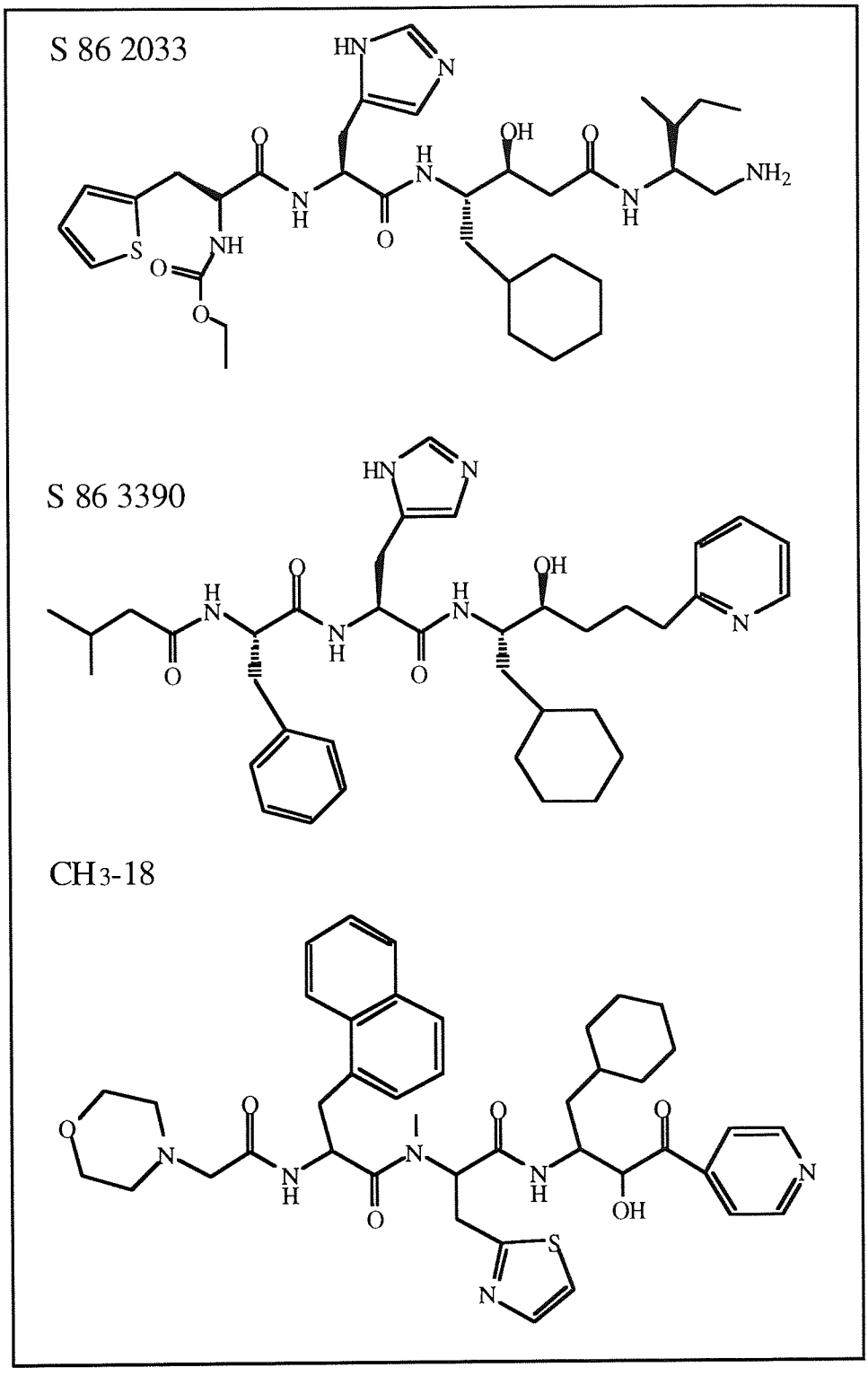
1.8.3 RENIN INHIBITORS

Renin inhibitors are a class of peptidomimetic drugs (angiotensinogen derivatives with the leucyl-valine (Leu-Val) bond replaced) which are used in the treatment of hypertension and congestive heart failure. They act by inhibiting the action of renin, which is the first enzyme, and rate-limiting step in the RAS cascade (Figure 1.5). Although renin inhibitors are active *in vitro*, the low oral bioavailability and stability is a limiting factor in effective therapy [Greenlee, 1987].

Two renin inhibitors, S 86 2033 and S 86 3390 (Figure 1.6), inhibited cephalexin uptake into rabbit BBMV in a concentration-dependent and competitive manner, suggesting that they are substrates for the DTS [Kramer *et al.*, 1990a]. S 86 3390 had highest affinity for the transporter, with an IC_{50} value of 1.1 mM compared to 2.5 mM for S 86 2033 [Kramer *et al.*, 1990a]. S 86 3390 was transported across the membrane into the BBMV, indicating that renin inhibitors are substrates for the DTS. S 86 3390 uptake was stimulated in the presence of an inward H^+ gradient, consistent with the di-/tripeptide/H^+ cotransport system [Kramer *et al.*, 1990a]. S 86 3390 and S 86 2033 both inhibited photoaffinity labelling of the 127 kDa binding protein of the DTS [Kramer *et al.*, 1990a].

Transport of the renin inhibitor ({3S, 4S}-4-[*N*-morpholinoacetyl-(1-naphthyl)-L-alanyl-*N*-methyl-{4-pyridyl}-1-pentanone; CH_3-18) (Figure 1.6) in rat small intestine was found to be stimulated in the presence of an inward H^+ gradient and completely and competitively inhibited by tripeptides and tetrapeptides [Hashimoto *et al.*, 1994a]. Amino acids and dipeptides had not effect on transport of CH_3-18, which suggests that it is being transported by a system other than that for dipeptides [Hashimoto *et al.*, 1994a]. β-Lactam antibiotics inhibited transport of CH_3-18, indicating the possibility of a transport system exclusively for β-lactam antibiotics as previously suggested [Nicklin *et al.*, 1996] which does not transport dipeptides, but has an affinity for tri-and tetrapeptides. The *N*-terminal substituent was found to be more important for transport than that of the *C*-terminal, with the presence of a morpholino group in this region increasing uptake [Hashimoto *et al.*, 1994a]. Substituting a naphthyl group at the *N*-terminal position has also been shown to increase intestinal absorption of some renin-inhibitors in rat intestine [Hashiomoto *et al.*, 1994b]. A weak basic function or a sulphonamide group at the *N*-terminal, has also been shown to increase the bioavailability of some renin inhibitors [Rosenberg *et al., 1993*].

FIGURE 1.6 *Renin inhibitors*



1.9 PRODRUG TARGETING OF THE DTS

There are several examples of the conversion of drugs to di-/tripeptide derivatives to improve oral absorption, through targeting to the DTS. L-α-Methyldopa is an aromatic amino acid used in the treatment of hypertension [Scriabine, 1980]. L-α-Methyldopa is a substrate for the LNAA transport system [Hu & Borchardt, 1990], however is has a low oral bioavailability (25 %). This is thought to be due to low membrane permeability as a result of the presence of the α-methyl group which hinders transport *via* the LNAA carrier and metabolism by the enzyme dopa-decarboxylase [Hu & Borchardt, 1990]. The absorption of L-α-methyldopa has been increased by converting it to the dipeptide prodrug L-α-methyldopa-L-phenylalanyl-L-alanine, which is a substrate for the DTS [Tsuji *et al.*, 1990].

L-3, 4-Dihydroxyphenylalanine (L-Dopa) is the primary drug used to treat Parkinson's disease. However, oral-L-Dopa has a low bioavailability as a result of degradation (decarboxylation) in the gut wall. Conversion of L-Dopa to the tripeptide p glutamyl-L-dihydroxyphenylalanyl-L-proline (pGlu-L-Dopa-L-Pro) was carried out and its intestinal permeability in rats was investigated [Bai, 1995]. The tripeptide prodrug was stable in intestinal homogenates, and intestinal permeability was found to be similar to that of the dipeptide L-phenylalanyl-L-proline (L-Phe-L-Pro). Permeability was inhibited by 20 mM captopril, 80 mM Gly-Gly and 5 mM Gly-L-Pro and by 2 mM cephradine, indicating that pGlu-L-Dopa-L-Pro is a substrate for the DTS [Bai, 1995]. pGlu-L-Dopa-L-Pro is converted to L-Dopa by peptidases [Bai, 1995]. The above two examples illustrate how conversion of drugs into peptide-like structures can be used to increase absorption, *via* targeting to the DTS.

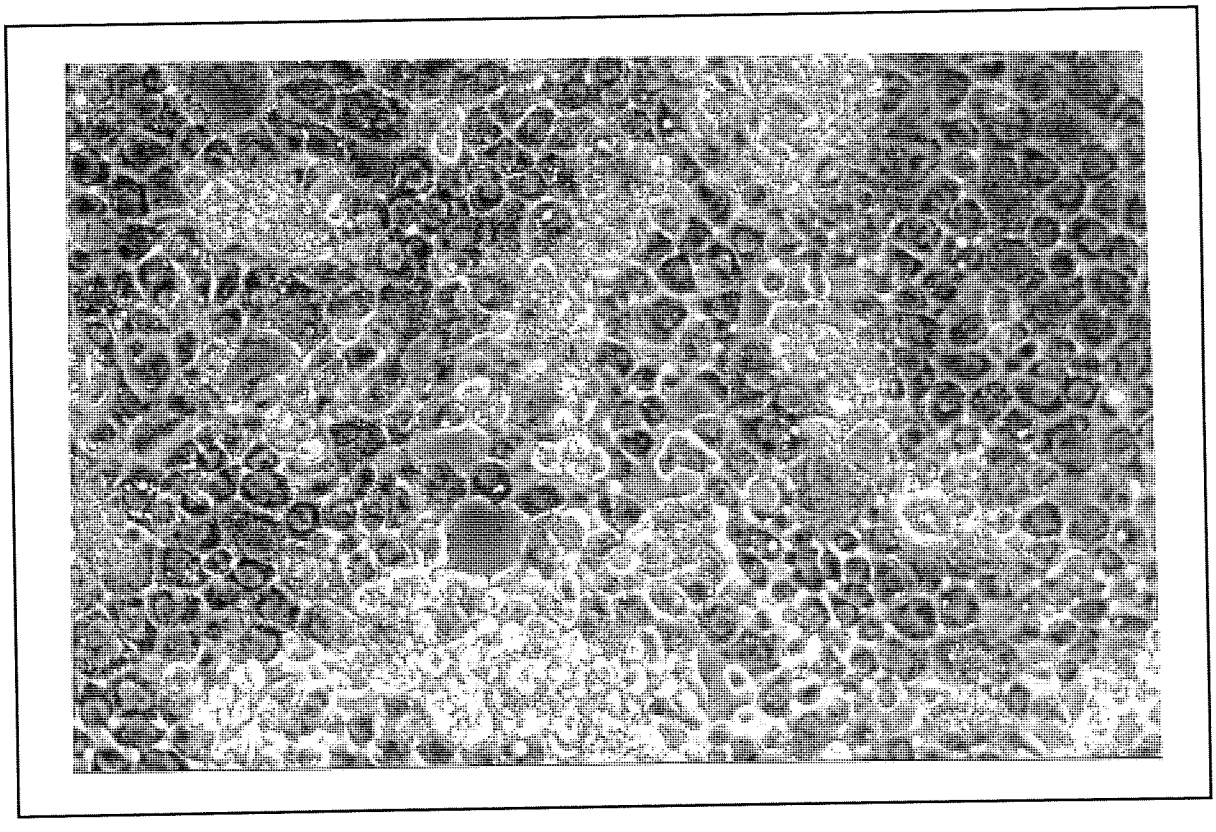
1.10 CACO-2 CELL LINE

1.10.1 INTRODUCTION

The Caco-2 cell line, which was establish using the explant culture technique, is an adenocarcinoma cell line originating from a well differentiated colonic tumour in a 72-year old Caucasian male [Fogh *et al.*, 1977]. When grown under standard culture conditions they spontaneously differentiate exhibiting enterocyte-like properties, expressing morphological and biochemical features of adult differentiated enterocytes [Pinto *et al.*, 1983: Zweibaum *et al.*, 1983, 1984]. They form polarised monolayers with apical and basolateral domains, with brush border microvilli extending perpendicular to the surface from the apical membrane [Pinto *et al.*, 1983]. The cells are separated by tight junction and form confluent monolayers, which appear, under a microscope (Figure 1.7) as an even spread of nuclei with a few empty spaces the size of 2 or 3 cells, which may be due to the presence of larger cells [Grasset *et al.*, 1984]. The transepithelial electrical resistance (TER) is approximately 300 Ω cm^2 [Artursson, 1991], which falls within the range documented for the small intestine and colon, 50-500 Ω cm^2 [Powell *et al.*, 1987]. The cells have been reported to form domes

when grown on an impermeable support after reaching confluency, consistent with a functionally polarised transporting epithelial monolayer [Pinto *et al.*, 1983]. The cells express certain enzymes which are associated with microvillus membranes. Alkaline phosphatase, aminopeptidase and sucrase-isomaltase are in comparable concentrations, 50, 10 and 50 % respectively, to those in the human small intestine [Pinto *et al.*, 1983]. Although Caco-2 cells have been shown to be fully differentiated by 15 d (growth on inserts), alkaline phosphatase activity continued to increase up to day 20 in culture [Pinto *et al.*, 1983: Hidalgo *et al.*, 1989b]. The activities of aminopeptidase and sucrase-isomaltase also increased after cell differentiation was completed [Pinto *et al.*, 1983]. γ-Glutamyltransferase [Blais *et al.*, 1987], lactase-phlorizin hydrolase and dipeptidylpeptidase IV are also expressed by Caco-2 cells [Hauri *et al.*, 1985]. The presence of brush border hydrolases and transport pathways *e.g.* bile acids (which will be discussed later), are properties of the distal ileum, while the transepithelial electrical resistance values are indicative to the colonic epithelium [Caro *et al.*, 1995]. Peptidase expression in Caco-2 cells will be considered further in Chapter 3.

FIGURE 1.7 *A Caco-2 cell monolayer grown on 24-well plates, 7 d post-seeding*



1.10.2 TRANSPORT SYSTEMS

Caco-2 cells express a number of transport systems which are present in intestinal enterocytes. A Na^+-dependent transport system for inorganic phosphate (P_i) is located at the apical membrane of Caco-2 cells, which displays characteristics similar to those found in intact small intestinal epithelium [Murer & Hildmann, 1981: Danisi *et al.*, 1984]. The

transport process involves two sodium ions to one phosphate ion and was found to decrease with increasing confluency, independent of cell age [Mohrmann *et al.*, 1986].

A bile-acid carrier system is present, which displays maximum expression after 28 d in culture [Hidalgo & Borchardt, 1990b]. Taurocholic acid (TA) is taken up by both the apical and basolateral membrane, but release is exclusively through the basolateral membrane, which is consistent of polarised delivery in normal enterocytes. Human ileal BBMV show carrier-mediated transport of bile acids, however, the jejunal BBMV do not [Barnard & Ghishan, 1987], suggesting that Caco-2 cells exhibit ileal characteristics. However, the carrier does differ in that it appears to be Na^+-dependent [Hidalgo & Borchadt, 1990b] and has a lower transport capacity than that of the enterocyte [Wilson *et al.*, 1990].

Caco-2 cells also possess an active transport system for the water-soluble vitamin riboflavine (RF) which plays a key role in carbohydrate, amino acid and lipid metabolism. The transport system is Na^+- and pH-independent, temperature and energy dependent, and can be inhibited by RF and RF analogues, indicating a carrier-mediated pathway [Said & Ma, 1994]. Furthermore, uptake of RF can be up- or down-regulated by substrate concentration in the growth media, which results in an increase or decrease in the number of transporters expressed at the apical membrane [Said & Ma, 1994].

Cyanocobalamin (Cbl) (Vitamin B_{12}) is taken up by Caco-2 cells in two forms, free or bound to an intrinsic factor (IF). The IF-Cbl binds to receptors at the apical membrane, with the complex then being internalised and the membrane bound receptors being recycled. Caco-2 cells also secrete a Cbl protein-binding factor from the basolateral membrane after 20 d in culture, which has similar properties to transcobalamin II (TCII). Cbl is transported through the cells in association with the TCII-like protein and exits exclusively *via* the basolateral membrane, being an example of receptor-mediated transcytosis [Dix *et al.*, 1990].

Caco-2 cell have also been shown to bind and internalise the epidermal growth factor (EGF) *via* a receptor-mediated process at both the apical and basolateral membranes [Hidalgo *et al.*, 1989a]. The receptor was found to occur in a ratio of 1:2.5 at the apical and basolateral membranes, respectively. In isolated human intestine and colonic cells, EGF binding in 17-week old foetuses was lower than in 12-week old foetuses [Pothier & Menard, 1988]. A similar process was observed with Caco-2 cells where binding of EGF decreased with the age of the monolayers [Hidalgo *et al.*, 1989a].

Caco-2 cell monolayers have been found to express an active transport system for monocarboxylic acid drugs. Benzoic acid is transported *via* a carrier-mediated transport system, which is energised by an inwardly directed proton gradient across the apical membrane [Tsuji *et al.*, 1994]. Salicylic acid transport is pH-dependent, comprising of a saturable and non-saturable process. Benzoic and acetic acid inhibited transport of salicylic acid whereas dicarboxylic acids, had no effect [Takanaga *et al.*, 1994], indicating that Caco-2 cells express a carrier-mediated transport process for monocarboxylic acids.

A Na^+-dependent sugar transport system, which is sensitive to both phlorizin and phloretin, as is the glucose transport system normally found in brush-border membranes of 16-22 week old human foetal colons, has been located in Caco-2 cells [Blais *et al.*, 1987: Riley *et al.*, 1991]. The family of glucose transporters is comprised of a series of proteins known as GLUT1-GLUT5. These proteins have identical secondary structures and are predicted to span the lipid bilayer 12 times, with an amino and carboxyl termini exposed at the cytoplasmic face. Caco-2 cells were found to have GLUT3 localised predominately at the apical membrane, which is thought to mediate the transport process. GLUT1's location is mainly at the basolateral membrane, and GLUT5 is found predominately at the apical membrane but also present in both the basolateral and intracellular membranes [Harris *et al.*, 1992]. GLUT5 was only detected in approximately 40 % of the fully differentiated Caco-2 cells, suggesting that under the experimental conditions the cells are not uniformly differentiated. However, this may also be due to the presence of phenotypically distinct cell types [Harris *et al.*, 1992]. The concept of different phenotypes existing within a population has also been observed with respect to enzymatic expression in Caco-2 cells (Chapter 3, section 3.) [Howell *et al.*, 1993].

Na^+-H^+ exchanger, which regulates intracellular pH (pH_i) is present exclusively on the basolateral membrane of Caco-2 cells. Na^+-H^+ exchanger is highly sensitive to amiloride (K_i 3.2 μM) and also has a cytoplasmic H^+ modifier site. The Na^+-H^+ exchanger was kinetically inactivated at resting pH_i (7.35 ± 0.02). Cell shrinkage and second messenger pathways which normally regulate Na^+-H^+ exchangers in other cell systems, had no effect in Caco-2 cells [Watson *et al.*, 1991]. The Na^+-H^+ exchanger has been found to be sensitive to serum, which regulates the activity by a F-actin-dependent post-translational mechanism [Watson *et al.*, 1992]. Caco-2 cells have also been shown to express an H^+-K^+-ATPase, which contributes to regulating pH_i [Abrahamse et al., 1992] and has been suggested to play an indirect role in peptide transport [Thwaites *et al.*, 1993g].

Caco-2 cells secrete lipoprotein particles in a polarised manner, indicating that they may be useful to study drug delivery to the lymphatic system [Hughes *et al.*, 1987]. Transport carriers for iron [Alvarez-Hernandez *et al.*, 1991], calcium [Guiliano & Wood, 1991] and folate [Vincent *et al.*, 1985] are also present. An active-transport system, which is involved in glutathione conjugate secretion from both plasma membranes is also present [Oude Elferink e*t al.*, 1993].

1.10.3 AMINO ACID TRANSPORT IN CACO-2 CELLS

Numerous amino acid transporters have been identified in the Caco-2 cell line. Grasset was the first to demonstrate this using electrophysiological techniques for the transport of L-Ala [Grasset *et al.*, 1984]. L-Ala transport exhibited temperature sensitivity, suggesting a carrier-mediated process [Cogburn *et al.*, 1991]. Competition studies revealed that L-Ala shares a Na^+-dependent carrier system in the apical membrane with L-Glu, which produced competitive inhibition [Souba *et al.*, 1992].

L-Ala transport has been shown to be driven by a proton electrochemical gradient even in Na^+-free media [Thwaites *et al.*, 1994b]. L-Ala was found to cause a marked cytosolic acidification consistent with proton/amino acid symport. Transport was found to be rheogenic and the stoichiometry is likely to be 1:1 [Thwaites *et al.*, 1994b]. Competition studies showed that L-Pro, α-aminoisobutyric acid and β-Ala are substrates for the L-Ala/proton symport [Thwaites *et al.*, 1994b]. However, L-valine (L-Val) and L-serine (L-Ser) are not substrates and likely to be transported by one of the other Na^+-dependent and Na^+-independent transporters for which L-Ala is a substrate [Stevens *et al.*, 1984: Christensen, 1990].

β-Ala flux from apical to basolateral surfaces was found to be absent even in the presence of Na^+ despite the accumulation of β-Ala across both membranes by a Na^+-dependent processes [Thwaites *et al.*, 1993e]. However, in Na^+-free conditions absorptive flux of β-Ala is observed, which is stimulated upon apical acidification (pH 6.0). Basolateral accumulation of β-Ala is absence in Na^+-free conditions, suggesting that the basolateral transport system is Na^+-dependent. Absorptive β-Ala flux (Na^+-free conditions) with an acidified apical solution (pH 6.0) displays saturation kinetics and is competitively inhibited but L-Ala and Gly, but not by L-Val and L-Ser [Thwaites *et al.*, 1993e]. Intracellular pH fell when 20 mM β-Ala was introduced to the apical solution, consistent with proton-coupled transport, and providing direct evidence for amino acid-stimulated proton influx across the apical membrane of Caco-2 cells [Thwaites *et al.*, 1993e].

The transport of Gly across Caco-2 cells monolayers has been shown to involve two processes [Smith *et al.*, 1991]. There is an active transport system which is unidirectional form the apical to basolateral regions, and a slower non-active process which is bi-directional and due to passive diffusion. Inhibiting the enzyme gamma-glutamyl transferase, results in an inhibition of Gly transport *via* the active process, indicating an involvement of the enzyme with this system [Smith *et al.*, 1991].

A transport carrier for the LNAA, L-Phe, has also been located in Caco-2 cells [Hidalgo & Borchardt, 1990a: Hu & Borchardt, 1992]. L-Phe transport was temperature-dependent, having an activation energy (E_a) for transcellular transport of 50.18 kJ mol^{-1} and was uneffected by a reverse gradient. The process was saturable with a 75 % reduction in transport seen in the presence of excess L-Phe, providing evidence for a carrier-mediated process. The transporter was Na^+-dependent as indicated by a 33 % decrease in transport observed in the presence of ouabain. Inhibition was seen with LNAAs (L-Leu and L-Tyr) and cationic amino acids (L-lysine {L-Lys} and L-His), but not with small neutral and acidic amino acids [Hidalgo & Borchardt, 1990a]. A preference for the L-isomer was also observed [Hidalgo & Borchardt, 1990a]. Apical to basolateral transport was considerably faster than that of basolateral to apical [Hu & Borchardt, 1992]. Initial uptake at both membranes was found to be saturable with K_m values for apical and basolateral uptake of 2.7 mM and 0.18 mM respectively, however, initial efflux rates were non-saturable. Different processes either ultising different carriers or combinations of carriers appear to be

responsible for uptake by the apical membrane and basolateral membrane and also efflux from the later. The basolateral efflux appears to be the rate-limiting step for transcellular transport [Hu & Borchardt, 1992].

L-α-Methyldopa is an aromatic amino acid used to treat hypertension which is transported *via* the LNAA carrier in Caco-2 cells [Hu & Borchardt, 1990]. Transport was concentration-, glucose-, pH- and temperature-dependent. Inhibition was observed with metabolic inhibitors and by L-Phe, an amino acid showing high affinity for the LNAA carrier [Hu & Borchardt, 1990].

A Na^+-dependent, acidic amino acid transport system, which is responsible for the transport of L-aspartic acid (L-Asp) and L-Glu is expressed by Caco-2 cells [Nicklin *et al.*, 1995]. The transport system which bears similarities to that found in the human jejunum [Rajendran *et al.*, 1987] is highly saturable (> 95 % at 50 M) with a major active component superimposed onto a minor passive (diffusional) component. Transport displays vectorial, pH- and temperature-dependent characteristics [Nicklin *et al.*, 1995]. Competition studies revealed that close analogues such as L-Asp, D-aspartic acid (D-Asp) and L-Glu are potent inhibitors, while analogues of L-Asp which possessed homologous extension of the methylene chain showed no interaction [Nicklin *et al.*, 1995].

A transport system for the imino acid L-Pro has also been located in Caco-2 cell monolayers. L-Pro uptake is comprised of an active component, which is responsible for two thirds of the observed flux over a nanomolar concentration at 37°C, and a passive component [Nicklin *et al.*, 1992a]. The active transport is saturable, showing temperature (E_a = 93.5 kJ mol^{-1}) and pH-dependence, with uptake increasing dramatically at pH 5.0 compared to that at pH 7.4 [Nicklin *et al.*, 1992a]. A higher degree of Na^+-dependency was observed for L-Pro uptake compared to that for L-Phe. When protein synthesis (cycloheximide), Na^+/K^+-ATPase (ouabain) or cellular metabolism (sodium azide) are inhibited, the efficiency of the carrier is greatly reduced [Nicklin *et al.*, 1992a]. L-Ser, L-Ala and α-aminoisobutyric acid produced potent inhibition of L-Pro uptake, whereas Gly, D-Pro and γ-aminoisobutyric acid were only moderate inhibitors [Nicklin *et al.*, 1992a]. No or very little inhibition was seen with aromatic or branched amino acids (*e.g.* L-Val, L-Phe, and L-Leu).

The Na^+-dependency of the L-Pro carrier system is being questioned, as L-Pro transport across Caco-2 cell monolayers was enhanced by acidification of the apical solution in both Na^+-free and Na^+-containing conditions [Thwaites *et al.*, 1993f]. pH-dependency of transepithelial transport and intracellular accumulation has been demonstrated in both the presence and absence of Na^+. The pH-dependency is a result of coupling of H^+ flow to L-Pro transport, as seen by the marked acidification of the cytosol with administration of L-Pro into the apical environment [Thwaites *et al.*, 1993f].

1.10.4 DTS IN CACO-2 CELLS

All of the investigation into dipeptide absorption/transport in Caco-2 cells have involved the use of the stable dipeptide probe Gly-Sar or cephalosporins. Gly-Sar transport has been shown to be rheogenic, stimulating an inward I_{sc} in voltage clamped Caco-2 cells [Thwaites *et al.*, 1993g]. The change in I_{sc} is observed under Na^+-free conditions, with an acidic apical media (pH 6.0) providing evidence of a di-/tripeptide/H^+ symport at the apical membrane [Thwaites *et al.*, 1993g]. This agrees with previous studies using BBMV and amphibian intestine [Boyd & Ward, 1982: Ganapathy *et al.*, 1984: Ganapathy & Leibach, 1985]. The stoichiometry of the dipeptide/H^+ symport is estimated to give a value of dipeptide to proton flux of 1:3 [Thwaites *et al.*, 1993g].

Transport and intracellular accumulation of Gly-Sar in Caco-2 cells from the apical to the basolateral was found to be optimal when the apical medium was at pH 6 (basolateral at pH 7.4) [Thwaites *et al.*, 1993b], therefore in the presence of a proton gradient. This was also found to be true for transport from the basolateral to apical region (basolateral pH 6.0, apical pH 7.4). Both transport and intracellular accumulation were found to be saturable and susceptible to competition [Thwaites *et al.*, 1993b]. Using cells preloaded with BCECF, a pH-sensitive fluorescent dye, an increase in cytosolic acidification was seen when 20 mM Gly-Sar was added to either the apical (pH 6.0) or basolateral media (pH 6.0). This is consistent with H^+-coupled dipeptide transport at both apical and basolateral membranes. Different transport rates across the two membranes suggest that the apical and basolateral transporters may differ [Thwaites *et al.*, 1993b]. Basolateral application of 20 mM Gly-Sar when the pH of the media was 7.4, failed to cause cytosolic acidification [Thwaites *et al.*, 1993a]. Transport and accumulation were inhibited when co-administered with 20 mM Gly-Sar or 20 mM cephalexin [Thwaites *et al.*, 1993a]. Interestingly, cephalexin caused a marked cytosolic acidification when presented at both the apical (pH 6.0) and basolateral (pH 7.4) membranes [Thwaites *et al.*, 1993a]. Transport of Gly-Sar and cephalexin across the basolateral membrane, although sharing common characteristics, also display distinct features, indicating the possible presence of more than one exit mechanism at the basolateral membrane [Thwaites *et al.*, 1993a].

The dipeptide L-Val-L-Val (20 mM) has also been shown to produce a marked intracellular acidification when perfused at the apical membrane, illustrating H^+-coupled dipeptide transport [Thwaites *et al.*, 1993c]. L-Val-L-Val inhibited pH-dependent apical to basolateral Gly-Sar transport and accumulation, confirming that the two dipeptides share a common transport system [Thwaites *et al.*, 1993c].

Gly-Sar transport and accumulation were also found to be inhibited by a range of compounds, including the dipeptides Gly-Gly, L-Leu-L-Leu, Gly-Gly-Gly, and the cephalosporins, cefadroxil, cefazolin and cephalexin [Thwaites *et al.*, 1994a]. These results were found to be similar to those reported in rabbit intestinal BBMV [Ganapathy *et al.*, 1984]. The ability of compounds to inhibit Gly-Sar transport/accumulation appear to correlate to their ability to induce intracellular acidification [Thwaites *et al.*, 1994a]. Gly-L-

Pro, Gly-L-Leu, Gly-L-Phe, carnosine and L-Val-L-Val all induced H+ flow into the cells, consistent with dipeptide H^+ cotransport [Thwaites *et al.*, 1994a]. D-Leucyl-D-leucine (D-Leu-D-Leu) failed to inhibit Gly-Sar transport and induce proton flow, indicating a stereoselective preference for the transport system [Thwaites *et al.*, 1994a]. The amino acid, L-Leu, also failed to inhibit transport and induce proton flow, as previously shown with L-Val [Thwaites *et al.*, 1993c], illustrating that the action is a dipeptide-specific effect [Thwaites *et al.*, 1994a].

Measurement of Gly-Sar accumulation in Caco-2 cells demonstrated that the dipeptide is accumulated in the cells against a concentration gradient [Brandsch *et al.*, 1994]. After a 30 min incubation the intracellular concentration of Gly-Sar was 142 ± 3 μM, with the concentration of the dipeptide in the apical media being 10 μM, providing clear evidence of uphill transport energised by an inward directed H^+ gradient [Brandsch *et al.*, 1994]. Gly-Sar transport across Caco-2 cells was found to be composed of an active process *via* the DTS and a passive diffusional component, as complete inhibition was not seen at 4°C, in Na^+-free conditions or in the presence of the protonophore [Gan *et al.*, 1994].

1.10.5 PEPTIDE AND PEPTIDOMIMETIC DRUG TRANSPORT IN CACO-2 CELLS

The absorption of ACE-inhibitors, and β-lactam antibiotics in Caco-2 cell monolayers will be discussed in Chapter 6.

TRH (pGlu-His-Pro-NH_2; TRH) is a hypothalamic tripeptide which stimulates thyrotrophin release. It has been shown to be absorbed by the GI tract, with oral administration (8 mg) producing small progressive increments in serum levels of tetraiodothyronine (T_4), which is released as a result of TRH [Haigler *et al.*, 1972]. This indicates that sufficient TRH is absorbed when administered orally, in order to elicit a physiological response.

The oral bioavailabilities of TRH in dogs, humans and rats are 12.6, 2 and 1.5 %, respectively. In beagle dogs, the oral bioavailability of 12.6 % was reduced to 6 % after ingestion of a meal, indicating that dietary protein products from the meal are competing with the TRH for transport. An *in vitro* experiment using everted sacs of rat intestine has shown that TRH uptake is concentration-dependent, vectorial and reduced by the presence of di- and tripeptides, suggesting an active carrier-mediated process *via* the DTS [Yokohama *et al.*, 1984].

TRH transport across Caco-2 cells has been found to have a large passive component (≥ 70 %), with a minor carrier-mediated pathway *via* the DTS [Nicklin *et al.*, 1991]. Uptake was found to be concentration-, pH- (maximum uptake seen at pH 8 when TRH is uncharged), and temperature-dependent [Nicklin *et al.*, 1991]. Cephalexin (10 mM) caused a 21.5 ± 3.1 % and 42.1 ± 2.6 % reduction in TRH transport and uptake, respectively [Nicklin *et al.*, 1991]. At 4 °C no inhibition was seen when co-administered with cephalexin, indicating a passive pathway [Nicklin *et al.*, 1991].

Contradictory evidence was found in rat and rabbit intestinal BBMV [Thwaites *et al.*, 1993h]. TRH accumulation was not affected by a Na^+ or H^+ gradient, and failed to reduced Gly-Sar transport, when co-administered in excess (46-fold), suggesting a passive paracellular process [Thwaites *et al.*, 1993h, 1994a]. These results were supported by similar findings in Caco-2 cells, whereby TRH transport across the monolayers was found to be similar to that of the paracellular marker, mannitol (1-4 % 4 h^{-1}) [Thwaites *et al.*, 1993d] Transport was unaffected by an acidic pH, excess cold TRH (20 mM) and was without saturation [Thwaites *et al.*, 1993d]. Therefore, suggesting that small, but pharmacologically significant amounts are achieved through passive, paracellular absorption of TRH [Thwaites *et al.*, 1993d].

The contradictory evidence for TRH transport in Caco-2 cells suggests the presence of different clones, which was highlighted by a subsequent study [Walter & Kissel, 1994]. TRH transport in Caco-2 cells of passage 89-99 shows an active carrier-mediated transport component with a passive pathway [Walter & Kissel, 1994], which is in agreement with the in vitro study [Yokohama *et al.*, 1984] and the previous Caco-2 study [Nicklin *et al.*, 1991]. TRH transport in Caco-2 cells at passage number 34 was found to be exclusively passive *via* a paracellular route [Walter & Kissel, 1994], agreeing with the BBMV study [Thwaites *et al.*, 1993e] and Caco-2 cell work [Thwaites *et al.*, 1993d, h]. Therefore, the conflicting results can be explained by the heterogeneity in characteristics shown by Caco-2 cells of differing origin and passage number [Walter & Kissel, 1994].

Bestatin [(2*S*, 3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucine], which is a modified dipeptide containing an unusual amino acid [Suda *et al.*, 1976], has been shown to have enhance the immune response and suppresses growth and metastasis of cancer, therefore displaying anticancer activity [Ebihara *et al.*, 1986]. Bestatin has been shown to inhibit cephradine uptake into rabbit BBMV in the presence or absence of an inward H^+ gradient, by a competitive mechanism (K_i = 0.47 mM), indicating absorption is *via* the DTS [Tomita *et al.*, 1990]. This was confirmed in a subsequent study, where bestatin uptake was stimulated by an inward H^+ gradient and an interior-negative membrane potential [Inui *et al.*, 1991]. At a 1 mM bestatin concentration, 50 % of the uptake was attributed to membrane binding [Inui *et al.*, 1991]. Cephalosporins and dipeptides inhibited bestatin uptake into the BBMV, indicating an active transport mechanism *via* the DTS [Inui *et al.*, 1991]. Bestatin uptake into Caco-2 cells was found to be pH-dependent at the apical surface, and subject to inhibition by a range of dipeptides and cephradine, indicating an active uptake process *via* the DTS, as previously reported [Saito & Inui, 1993].

Thrombin inhibitors are a class of drugs used in the treatment of inherited thrombotic disorders and antithrombic therapy. These are peptidomimetic drugs which are derived from L-aspartyl-D-phenylalanine (L-Asp-D-Phe). The transport of five structurally related thrombin inhibitors which differ in their substitution of the L-aspartyl-β-carboxyl group (Figure 1.8), across Caco-2 cell monolayers and the oral bioavailabilities in rats, were investigated [Walter *et al.*, 1995].

The transport rates differed for the five compounds with permeability coefficients ranging from 0.126 x 10^{-6} cm s^{-1} (compound 1) to 0.031 x 10^{-6} cm s^{-1} (compound 2). Interestingly, compound 2 had the highest Log P value (1.6), yet the lowest transport rate across the monolayers, suggesting that there is no correlation between Log P and transport across the monolayer [Walter *et al.*, 1995]. Compounds 2-5 were found to be transported *via* the paracellular pathway, whereas compound 1, with an oral bioavailability in rats of 3.8 % was found to involve an active process [Walter *et al.*, 1995]. Compound 1, which has a free carboxyl- and an amide-group had higher permeability across Caco-2 cells and higher bioavailability in rats compared to the other compounds. Gly-L-Pro (27 mM) and cephradine (27 mM) inhibited the transport of compound 1 by 31 % and 51 % respectively, across Caco-2 cell monolayers, providing evidence that compound 1 is a substrate for the DTS [Walter *et al.*, 1995]. The transport of compound 1 across Caco-2 cell monolayers was temperature- and concentration-dependent, with kinetic parameters; K_m = 1.67 ± 0.62 mM and V_{max} = 26.5 ± 4.8 pmol min^{-1} mg $protein^{-1}$. Transport of compound 1 was *via* a saturable carrier-mediated process (DTS), which accounted for 50 % of transport, and a non-saturable passive component, with a k_d of 4.87 ± 0.41 x 10^{-3} nmol min^{-1} mg $protein^{-1}$ mM^{-1} [Walter *et al.*, 1995]. The carboxyl group is esterified in compounds 2-5, and thus, may explain the lack of active transport for these compounds.

FIGURE 1.8 *Chemical structures of the thrombin inhibitors (1-5)*

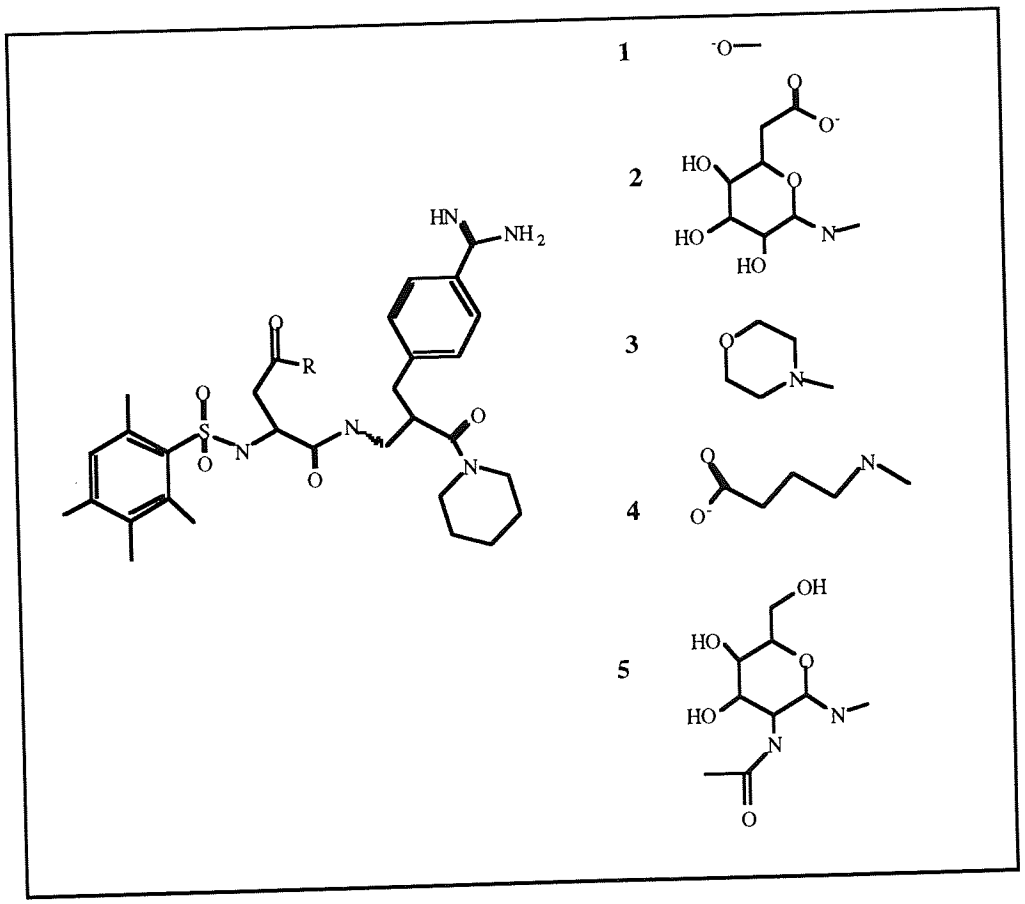


TABLE 1.3 *Summary of compounds other than di-/tripeptides that are substrates for the DTS*

Class of compound	Compound	References
ACE-inhibitors	Benazepril	Yee & Amidon, 1990: Kim *et al.*, 1994
	Captopril	Duchin *et al.*, 1982: Singhvi *et al.*, 1982: William & Sugerman, 1982: Hu & Amidon, 1988
	Enalapril	Friedman & Amidon, 1989b: Yuasa *et al.*, 1994: Swaan *et al.*, 1995
	Enalaprilat	Yuasa *et al.*, 1994: Swaan *et al.*, 1995
	Lisinopril	Friedman & Amidon, 1989a: Swaan *et al.*, 1995
β-lactam antibiotics	Ampicillin	Oh *et al.*, 1992
	Amoxicillin	Oh *et al.*, 1992
	Cefaclor	Okano *et al.*, 1986a: Sinko & Amidon, 1989
	Cefatrizine	Sinko & Amidon, 1989
	Cefadroxil	Nakashima *et al.*, 1984a, b: Okano *et al.*, 1986a: Sinko & Amidon, 1989
	Cefixime	Inui *et al.*, 1988: Yoshikawa, *et al.*, 1989: Kramer *et al.*, 1995
	Ceftibuten (cis isomer)	Inui *et al.*, 1988: Yoshikawa, *et al.*, 1989: Sugawara *et al.*, 1991
	Cephalexin	Nakashima *et al.*, 1984a, b: Okano *et al.*, 1986a: Tamai *et al.*, 1987: Sinko & Amidon, 1989: Kramer et al., 1992: Kramer *et al.*, 1995
	Cephradine	Nakashima *et al.*, 1984b: Okano *et al.*, 1986b: Sinko & Amidon, 1989: Iseki *et al.*, 1989: Tomita *et al.*, 1995
	Cyclacillin	Nakashima *et al.*, 1984a, b
	Penicillin-G	Poschet *et al.*, 1996
	S-1090	Muranushi *et al.*, 1995

TABLE 1.3 *Summary of compounds other than di-/tripeptides that for substrates for the DTS, continued,*

Renin inhibitors	S 86 2033	Kramer *et al.*, 1990a
	S 86 3390	
	CH_3-18	Hashimoto *et al.*, 1994a
Thrombin inhibitors	Compounds 1-5 (Figure 1.8)	Walter *et al.*, 1995
Tripeptide produgs	pGlu-L-Dopa-Pro	Bai, 1995
	α-Methyldopa-L-Phe	Tsuji *et al.*, 1990
Other compounds	Bestatin	Tomita *et al.*, 1990: Saito & Inui, 1993
	TRH	Nicklin *et al.*, 1991: Thwaites *et al.*, 1993d, h: Walter & Kissel, 1994

Note the ACE-inhibitors and β-lactam antibiotics that are substrates for the DTS in the Caco-2 cell model are not included in the above table and are mentioned in Chapter 6.

1.10.6 SUITABILITY OF THE CACO-2 CELL LINE AS A DRUG TRANSPORT MODEL

The Caco-2 cell line, has been designated the most relevant *in vitro* model for studies investigating the differentiation and regulation of intestinal absorptive-digestive functions [Rousset, *et al.*, 1986].

Caco-2 cells when grown on plastic dishes or nitrocellulose filters have been shown to develop morphological characteristics of normal enterocytes. After 16 days in culture, Caco-2 cells grown on collagen coated polycarbonate membranes displayed similar characteristics to that of the columnar epithelium of the small intesine [Hidalgo *et al.*, 1989b]. The TER value was 173.5 Ω cm^2 after reaching confluency and remained unchanged through to d 17 [Hidalgo *et al.*, 1989b], which is consistent with values recorded for the GI tract [Powell *et al.*, 1987]. The uptake and permeability of horseradish peroxidase was similar to that seen in the intestinal epithelia layer, confirming the suitability of the Caco-2 cell system as a valuable transport model for the small intestine [Hidalgo *et al.*, 1989b].

The suitability of the Caco-2 cell system as a drug screening model for passively absorbed compounds, was assessed [Artursson & Karlsson, 1991]. A good correlation between apparent permeability coefficients in the Caco-2 model and absorption in humans after oral administration was achieved [Artursson & Karlsson, 1991]. Drugs that are completely absorbed in humans produced permeability coefficients > 1 x 10^{-6} cm s^{-1}, drugs that were 1-99 % absorbed had values of 0.1-1.0 x 10^{-6} cm s^{-1}, and drugs that were < 1% absorbed had permeability coefficients of $\leq$ 1 x 10^{-7} cm s^{-1} [Artursson & Karlsson, 1991].

The type of permeable support that the cells were grown on, was found to influence the transport of certain compounds across the monolayers [Nicklin *et al.*, 1992b]. The bile acid TA, was used as the model compound. The transport rate of TA was found to be higher in Caco-2 cells grown on nitrocellulose inserts compared to aluminium ones, 59.3 $\pm$ 4.1 ng 4 h^{-1} $insert^{-1}$ compared to 29.7 $\pm$ 4.1 ng 4 h^{-1} $insert^{-1}$, respectively [Nicklin *et al.*, 1992b]. Basolateral transport characteristics of TA were also different for the two inserts. The aluminium oxide inserts had a low adsorption potential, making them suitable for transport studies involving high molecular weight and lipophilic compounds. The aluminium oxide inserts had a considerably lower cell number compared to the nitrocellulose inserts. However, the aluminium oxide inserts had a higher TER, 871 $\pm$ 149 Ω cm^2 compared to 513 $\pm$ 32 Ω cm^2 for the nitrocellulose inserts. This is probably due to the selection of different sub clones of the cells by the different inserts [Nicklin *et al.*, 1992b]. This, illustrates that the selection of the permeable support must be considered when undertaking transport studies and also when comparing to previous work.

The Caco-2 cell model was assessed as an *in vitro* intestinal absorption and metabolism model using a number of compounds transported *via* different processes [Gan *et al.*, 1994]. Results showed that it is an excellent tool for distinguishing passive diffusion from carrier-mediated active transport, and also for identifying paracellular and transcellular

transport pathways [Gan *et al.*, 1994]. However, recently the suitability of the Caco-2 cell line as an intestinal drug absorption model has been questioned. There is no doubt that for passively absorbed drugs, the Caco-2 cell line is an excellent model, as a good correlation between permeability coefficients in Caco-2 cells and oral bioavailability in humans has been demonstrated [Artursson & Karlson, 1991]. However, several studies investigating the transport of actively absorbed drugs have found that it is under-estimated in the Caco-2 cell model.

The barrier function of the tight junctional complexes in the Caco-2 cell model have been reported to be comparable to that of the intact human intestine [Artursson *et al.*, 1993]. The permeability of PEGs was dependent upon the molecular weight, with a cut-off point being seen with molecular weights of 400-500 g mol^{-1}, which is in agreement with previous results [Artursson *et al.*, 1993]. This highlights the usefulness of the Caco-2 cell model for drug absorption studies.

SQ-29852, an ACE-inhibitor with a bioavailability of 67 % in humans [Foley *et al.*, 1988], was found to have poor transport across Caco-2 cells [Nicklin *et al.*, 1996]. Its calculated permeability coefficient was less than that for the hydrophilic marker molecule, PEG_{4000} (paracellular transport), when using the correlation for passively absorbed drugs [Artursson & Karlsson, 1991], giving a predicted oral bioavailability in man of > 1 % [Nicklin *et al.*, 1996]. Another study using a larger number of compounds found that permeability of lisinopril, captopril, SQ-29852, and cephalexin across Caco-2 cells were low, predicting poor absorption in humans despite good absorption demonstrated *in vivo* [Chong *et al.*, 1996]. This clearly illustrates that for actively absorbed compounds *via* the DTS the Caco-2 model is under-estimating transport, which may possibly be due to an under expression of peptide transporters at the basolateral membrane in Caco-2 cells compared to the normal absorptive cells *in vivo*, or that the basolateral transport system is not functioning properly.

This finding was confirmed in a study comparing active and passive drug transport in Caco-2 cells to that in the human jejunum [Lennernas *et al.*, 1996]. Drugs such as naproxen, antipyrine and metoprolol, which are passively (rapidly) transported drugs, were found to have comparable permeability coefficients in Caco-2 and human jejunum [Lennernas *et al.*, 1996]. However, permeability of slower (passively) transported, hydrophilic drugs, such as terbutaline and atenolol were found to be 79- and 27-fold lower in Caco-2 cells compared to the human jejunum [Lennernas *et al.*, 1996]. L-Dopa, L-Leu and D-glucose, which are all active carrier-mediated compounds were found to have considerably slower transport rates in Caco-2 cells [Lennernas *et al.*, 1996]. The fact that atenolol, terbutaline and the actively absorbed compounds had lower permeability in Caco-2 cells may reflect that they are of colonic origin, and suggest that a scaling-up factor may be required for predicting absorption of carrier-mediated compounds [Lennernas *et al.*, 1996]. Therefore, although the Caco-2 cell model provides an excellent mechanistic tool for identifying and characterising transport

systems, its use in identifying drug candidates and predicting transport rates for compounds which are actively absorbed *via* the DTS, may be limited.

1.10.7 CLONES OF THE CACO-2 CELL LINE

The parental cell line Caco-2 appears to be morphological heterogeneous [Wilson *et al.*, 1990], with clones expressing different levels of enzymes and transport systems having differing characteristics. Sucrase activity and glucose utilisation were found to vary between clones of early passage (15-30) and those of late passage (110-120), indicating heterogeneity of the parent population [Zweibaum *et al.*, 1983]. Clones from passage 29 and passage 198 Caco-2 cells have been characterised [Chantret *et al.*, 1994: Mahraoui *et al.*, 1994]. The apical brush border was present in both clones as well as similar cell polarity, and enzyme expression (dipeptidyl-IV, aminopeptidase N and alkaline phosphatase). However, the glucose consumption, expression of sucrase-isomaltase and fructose transporter GLUT5, were different [Chantret *et al.*, 1994: Mahraoui *et al.*, 1994]. It has been suggested that the transport properties of monolayers are the sum of transport by the different clones in the population [Caro *et al.*, 1995].

The uptake of TA was compared in three Caco-2 cell clones, PD-7 and PF-11 from early passages and TC-7 from a late passage [Caro *et al.*, 1995]. All three clones formed well-differentiated monolayers, with apical brush border membranes [Caro *et al.*, 1995]. Mannitol (paracellular route) and testosterone (passive diffusion) transport observed in the clones were only slightly different when compared to the parental cell line, as were the transepithelial electrical resistance values [Caro *et al.*, 1995]. However, transport values for TA, which is *via* an active process, were considerably different, with V_{max}/K_m values of 0.037, 0.048, 0.060 and 0.178 for the parental cell line, PD-7, PF-11 and TC-7, respectively [Caro *et al.*, 1995]. Caco-2 cells, under certain conditions, have been shown to express different enzyme systems including cytochrome P450 mono-oxygenases [Boulenc *et al.*, 1992: Rosenberg & Leff, 1993] and UDP-glucuronosyltransferases [Bjorge *et al.*, 1991] which are involved in the biotransformation of endogenous compounds and xenobiotics. TC-7 was found to be inducible to the P450IA1 enzyme (22-fold) and expressed a higher activity of UDP-glucuronosyltransferase, compared to the other clones tested [Caro *et al.*, 1995]. Therefore, TC-7 clone of the Caco-2 cell line is a suitable model for the intestinal disposition of drugs due to its enhanced transport and metabolic characteristics [Caro *et al.*, 1995].

1.10.8 P-GLYCOPROTEIN EXPRESSION IN CACO-2 CELL MONOLAYERS

P-glycoprotein (MDR1 gene product) is a membrane glycoprotein (170-180 kDa), which is associated with pleiotropic (multidrug) resistance (MDR) in tumour cells [Ford & Hait, 1990: Pastan & Gottesman, 1991]. P-glycoprotein, functions as an ATP-dependent drug-efflux pump and limits the active transport of lipophilic substrates such as vinblastine and vincristine, which are chemotherapeutic drugs originating from plants and microbial

sources [Dewick, 1989: Goldstein *et al.*, 1992]. Expression of P-glycoprotein, which is found localised in the apical membrane of the cells [Thiebaut *et al.*, 1989], have been reported to be present in epithelia of the GI tract [Cordon-Cardo *et al.*, 1990], where it secretes natural toxic compounds, which are normal constituents of the diet, and therefore limits absorption.

Western blotting and monoclonal antibody data have demonstrated the functional expression of P-glycoprotein in Caco-2 cells monolayers [Peters & Roelofs, 1992]. The cells have been shown to exhibit an intestinal absorptive villous enterocyte phenotype with respect to P-glycoprotein expression, which was accompanied by net secretory (basolateral to apical) transport of vinblastine, a typical substrate for the MDR pump [Hunter *et al.*, 1993a]. After treatment with MRK16 antibodies (monoclonal antibodies which recognise external epitopes of P-glycoprotein) vinblastine secretory flux was reduced indicating a direct correlation to apical P-glycoprotein expression. Competitive inhibition of vinblastine secretory flux was demonstrated with verapamil and nifedipine, whereas inhibition seen with dideoxyforskolin was non-competitive [Hunter *et al.*, 1993a].

As the Caco-2 cell model is used extensively to predict oral drug absorption, the high capacity P-glycoprotein efflux pump at the apical membrane may possibly alter drug absorption kinetics through secretory flux [Hunter *et al.*, 1993b]. The synthetic tripeptide *n*-acetyl-leucyl-leucyl-norleucine has been shown to be a substrate for P-glycoprotein [Sharma *et al.*, 1992], and therefore raises the possibility that peptide absorption may be limited by active export mediated by P-glycoprotein. A model peptide, acetamido-D-phenylalanyl-amide, has also been shown to a substrate for the efflux system, showing saturable transport that could be competitively inhibited by verapamil [Burton *et al.*, 1993]. The surfactants Cremophor EL and Polysorbate 80 inhibit P-glycoprotein in cancer cells, thus enhancing the uptake of chemotherapeutic agents [Woodcock *et al.*, 1990]. A similar effect was observed with acetamido-D-phenylalanyl-amide, with permeability through Caco-2 cells increasing when co-administered with the surfactants Cremophor EL and Polysorbate 80, which act by inhibiting the apically polarised efflux system [Nerurkar *et al.*, 1996].

1. 11 OLIGODEOXYNUCLEOTIDES

1.11.1 INTRODUCTION

Oligodeoxynucleotides (ODNs) are short lengths of single-stranded deoxyribonucleic acid (DNA) composed of complementary (antisense) sequences to a specific gene or its mRNA [for review see Akhtar & Juliano, 1991, 1992]. They offer exciting possibilities for modifying the expression of a particular gene without affecting the functions of others. Potential therapeutic applications for the treatment of diseases where undesirable gene expression occurs, such as in acquired immunodeficiency syndrome (AIDS) and cancer are being considered. ODNs are currently undergoing clinical trials for

treatment of leukaemias [McManaway *et al.*, 1990], viral infections [Agrawal & Tang, 1992], herpes simplex [Gao *et al.*, 1990] and human papilloma virus [Cowsert *et al.*, 1993].

1.11.2 MECHANISM OF ACTION

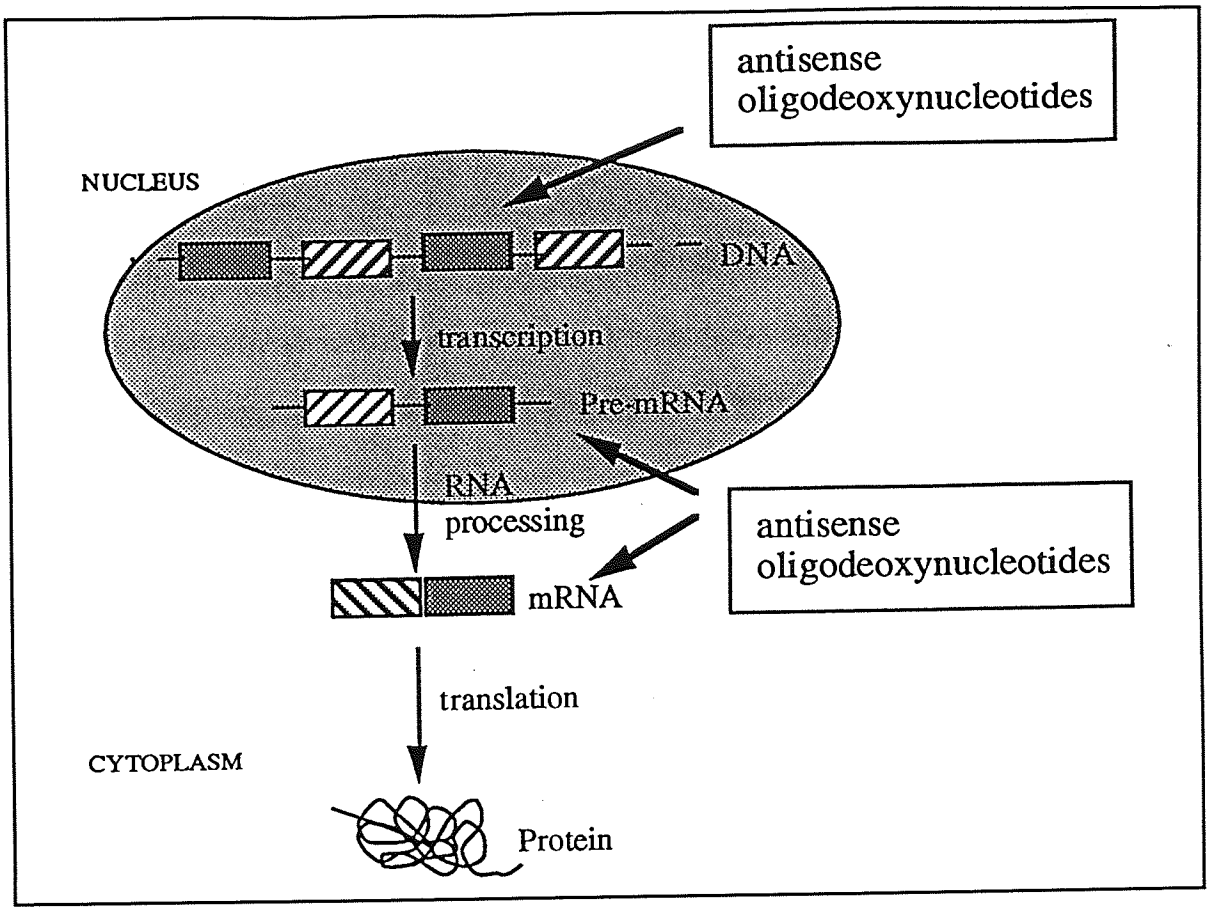
In order to understand how ODNs can modulate gene expression in living cells, the flow of genetic information within the cell must be considered. Genetic information is encoded by specific sequences (genes) within double-stranded helical DNA, which is composed of four nucleotide bases (adenine, thymine, guanine and cytosine) substituted around a sugar-phosphate backbone. Each gene codes for a specific protein. Transcription, copying the genetic code from the DNA to mRNA, is the first stage in the production of a protein, and takes place in the nucleus. The DNA double-helix unwinds and the sense strand is then transcribed (copied) by the enzyme RNA polymerase, which produces antisense pre-mRNA. The pre-mRNA, which codes for exons (coding sequences) and introns (non-coding sequences), is then modified, which is known as RNA splicing. This involves the removal of the non-coding sequences (introns) and rejoining of the exons to produce a mRNA. The mRNA then moves from the nucleus into the cytoplasm and the information it carries is translated by the ribosomes, from the 5'- end to the 3'- end, into the specific protein. The protein is responsible for producing the effect controlled by the gene.

ODNs can interfere with gene expression at each level of the process, from the DNA to the final product (see Figure 1.9). ODNs can bind by Hoogsteen binding (thymidine binds to adenine thymidine pairs, and protonated cytosine binds to guanine-cytosine pairs) to the sense sequence of chromosomal DNA to produce a triple helix [Moser & Dervan, 1987: Riordan & Martin, 1991]. This directly inhibits transcription and prevents the production of pre-mRNA. ODNs can also form Watson-Crick base pairing to the mRNA and therefore prevent translation and interfere with protein production through the following pathways [Kregenow *et al.*, 1995];

1) Disrupts RNA processing,
2) Stops translocation of the mRNA into the cytoplasm,
3) Interfere with the binding of the ribosomes to the mRNA,
4) Can impair the translation process itself,
5) By hybridising to the mRNA it creates a duplex which is a substrate for ribonuclease H (RNase H), which degrades the mRNA.

Some ODNs act by interfering with the metabolism of the cell, by binding to cellular proteins, which control homeostasis [Riordan & Martin 1991]. However, this is not ideal as specificity is compromised.

FIGURE 1.9 *Diagram showing the possible sites of action of ODNs (modified from Akhtar & Juliano, 1991)*



1.11.3 BIOCHEMISTRY OF ODNS

ODNs are short single strands (10 to 30 bases) of synthetic stretches of DNA or RNA complementary to specific genetic targets of mRNA. It has been estimated that an ODN sequence of 17 nucleotides will occur only once in the human genome, and therefore, ODNs are usually at least 15 bases long which provides sequence uniqueness [Uhlmann & Peyman, 1990]. The length of the strand is of great importance, shorter strands are easier and cheaper to synthesise but may bind to related sequences in non-target genes and therefore lack specificity. Longer strands have increased specificity but are more expensive and may not be taken up into the cells. Folding and looping of the longer ODNs may occur due to self-complementarity, when sequences on the same molecule match up, and therefore a compromise is needed.

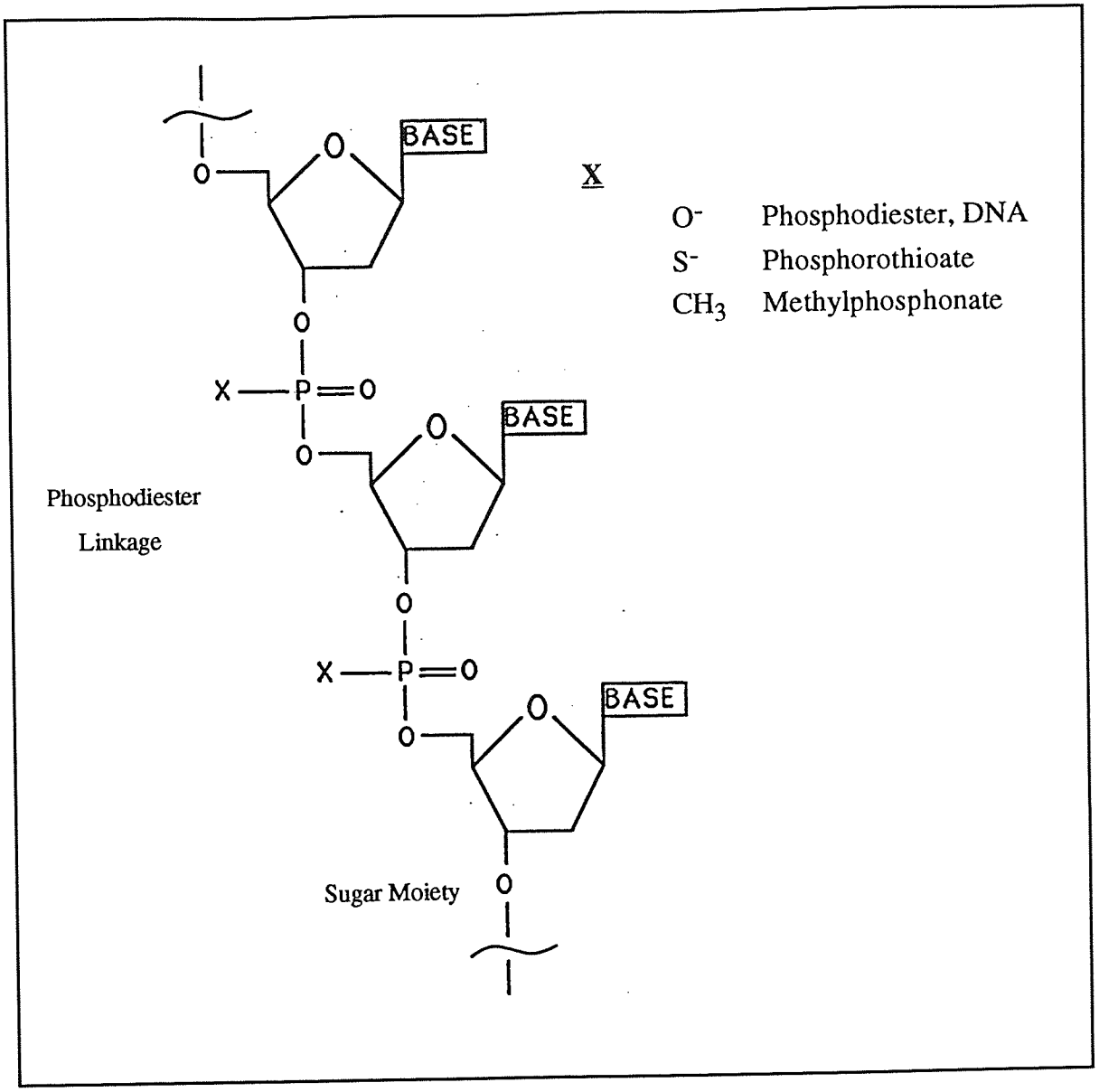
In order to create an ODN that can produce the greatest clinical effect, several criteria must be met. Firstly the ODN needs to be stable in both intra- and extra-cellular melieu; secondly the ODN must be able to reach the target site (nucleus or cytoplasm), therefore they must be able to gain access to the cell by transversing the cell membrane; thirdly, they must demonstrate specificity; and finally they must show little toxicity.

Initially, ODNs were prepared with unmodified phosphodiester (PO) linkages (Figure 1.10). Unmodified PO are subject to digestion by 3'- and 5'- exonucleases and endonucleases [Stein & Cheng, 1993] present in the cytoplasm and nucleus, resulting in their therapeutic application being limited. Several modifications have since been made to the PO linkage in order to increase the cellular stability of the ODN. These include replacing the oxygen with a methyl or sulphur group to produce a methyl phosphonate (MP) or phosphorothioate (PS) respectively, which has been shown to increase biological stability [Kregenow *et al.*, 1995]. Other modifications include changing the glycosidic linkage from the β to the α anomeric form, and capping/altering the 3'- and 5'- ends of the oligomer molecules. Examples of second-generation modifications are the production of a co-polymer consisting of PS and PO [Ghosh *et al.*, 1993], and replacing the deoxyribose phosphate backbone with a polyamide backbone composed of (2-aminoethyl)glycine units, to produce a peptide-nucleic acid analogue (PNA) [Nielsen *et al.*, 1993].

1.11.4 CELLULAR UPTAKE

The nature of ODNs, in that they are large and polyanionic, would suggest that they are not transported across cell membranes. However, Zamecnik and Stephenson in 1978, showed that, when antisense PO were added to culture media, inhibition of selective genes was achieved. This suggested that the PO were present inside the cells [Zamecnik & Stephenson, 1978]. Two independent groups later showed that PO are taken up into cells, probably *via* endocytosis involving a specific cell-surface-binding protein [Loke *et al.*, 1989: Yakubov *et al.*, 1989]. Both groups identified an 80 kDa receptor protein using different methods. Loke *et al.*, showed that binding of the PO to cultures of HL60 cells, could be blocked by excess PS but not MP, suggesting that the MP utilise a different receptor protein or mechanism. Both of these studies came under criticism, as Loke's group used a long incubation period (50 hours) and media containing 10 % foetal calf serum, yet no monitoring of the stability of the PO was carried out. Both groups did not remove the bound ligand at 4°C with proteolytic enzymes, this being an indicator of the involvement of a protein receptor.

FIGURE 1.10 *Structure of DNA ODN, showing modification in the phosphodiester linkage (X) (modified from Akhtar & Juilano 1991)*



PS are believed to also enter cells by endocytosis [Stein *et al.*, 1988: Marti *et al.*, 1989], and the fact that they compete with PO suggests that the same receptor is involved. As MP are neutral hydrophobic molecules they were originally thought to enter cells by passive diffusion [Miller *et al.*, 1981]. However, kinetic studies of the efflux of MP from artificial membranes (liposomes) produced similar values to those of PO and PS, suggesting that diffusion is not the method of cellular uptake [Akhtar *et al.*, 1991a]. This was confirmed in a subsequent study, that showed the uptake of MP into CHO cells occurred rapidly and the process was temperature-dependent, indicating an active mechanism [Shoji *et al.*, 1991]. Excess ATP had no effect on uptake and excess PO and cold MP only showed slight inhibition, suggesting that uptake of MP is non-specific and of a different route to that of PO. Absorptive or fluid phase endocytosis has been suggested as the mechanism of MP uptake, as the distribution of fluorescent labelled oligo was characteristic of localisation

within endosomal vesicles [Shoji *al.*, 1991], and MP bind strongly to lipid membranes [Akhtar *et al.*, 1991a]. Recently, uptake of PO in Rauscher Red 5-1.5 erythroleukemia cells was found to predominately be non-specific and did not appear to be receptor-mediated endocytosis [Wu-Pong et al., 1994], suggesting that the mechanism of ODN internalisation may be cell-line dependent.

1.11.5 INTRACELLULAR FATE OF ODNS

Once inside the endosomal compartment, the ODN has to be released into the cell cytosol for it to have a biological effect and reach its genetic target. It has been shown, using model liposomal compartments which have characteristic endosomal properties, that ODN diffusion across these membranes is slow and unlikely to be the exit mechanism [Akhtar *et al.*, 1991a]. Proteins present within the endosomal lipid bilayer are thought to facilitate the release of viruses [White *et al.*, 1983] and bacterial toxins [Farahbahksh *et al.*, 1987] through membrane destabilisation, and therefore this mechanism can not be ruled out for ODN release. Also, the constant fusing of endosomes to other vesicles may permit the release of ODNs *via* leakage.

The fate of ODNs once outside of the endosomal compartments is still undecided. Reports have shown that ODNs localise predominately in the cytosol [Cerruzzi *et al.*, 1990] and others show localisation is within the nucleus [Leonetti *et al.*, 1991]. Microinjection studies have shown that ODNs migrate to the nucleus [Chin *et al.*, 1990], however antisense activity is seen against the vesicular stomatitis virus whose replication cycle is entirely within the cytosolic fraction [Kulka *et al.*, 1989]. Therefore, it suggests that ODNs distribute in both the cytosol and the nucleus.

1.11.6 MODIFICATIONS TO INCREASE ODN DELIVERY

The effectiveness of the ODN relies on maximal delivery to the genetic target, therefore several strategies for improving delivery are being investigated. One approach being developed to increase cellular uptake is to conjugate the ODN to lipophilic groups or membrane destabilising moieties, these include cholesterol [Letsinger *et al.*, 1989], lipids [Shea *et al.*, 1990] and poly-L-lysine [Degols *et al.*, 1991]. This area will be further discussed in Chapter 7.

1.11.7 POTENTIAL APPLICATIONS FOR ODNS

Antisense ODN have a number of possible therapeutic applications for the future. The two areas receiving the most interest are the anticancer and antiviral fields. However, antisense ODNs have been used as an experimental treatment of malaria, and have been shown to inhibit smooth muscle proliferation [Simons *et al.*, 1992],which may suggest a possible role in the treatment of cardiovascular diseases.

The treatment of leukaemias, by targeting antisense ODNs to oncogenes and oncogene products, is an exciting possibility. In certain types of Burkitt's lymphomas

chromosomal changes involving the *c-myc* oncogene occur, resulting in abnormal mRNA, coding for intron sequences. ODNs targeted against the abnormal mRNA have been shown to inhibit cell growth and reduce c-myc protein expression [McManaway *et al.*, 1990], and therefore indicate a role for antisense ODNs in tumour therapy.

Another possible target is the multiple drug resistance (MDR-1) gene, which codes for P-glycoprotein. This is a membrane bound protein which acts as a drug efflux pump, and is thought to be over-expressed in drug resistant cells. These cells can not accumulate drugs intracellulary and therefore are resistant to conventional chemotherapy. Cells which displayed drug resistance to doxorubicin, when treated with antisense MP were found to have their sensitivity restored [Vasanthakumar & Ahmed, 1989]. This was achieved by blocking gene expression at the translation stage.

Antisense ODNs have also been targeted successfully against the herpes simplex and influenza viruses. The human immunodeficiency virus (HIV) is also a possible target for ODNs. Studies focusing on blocking the production of proteins vital for the viruses existence, are currently underway. The production of two HIV proteins, p15 and p24, have successfully been inhibited in infected cells using antisense ODN [Zamecnik *et al.*, 1986]. PS have also demonstrated antiviral activity and are thought to work by interfering with the enzyme systems of the virus.

1.12 OBJECTIVE OF THE THESIS

Several classes of orally administered peptidomimetic drugs such as the ACE-inhibitors and the β-lactam antibiotics have been shown to utilise the DTS to contribute, in part, to their overall absorption. The aim of the thesis is to provide information on the structural requirements needed for peptides and peptidomimetic compounds to interact with the DTS. The investigation of the structural requirements needed by a molecule to ensure maximum interaction with the DTS will be explored by the development and characterisation of a rapid uptake model system that can be used to screen a variety of structural analogues for interaction. The stereochemical and charge preference of the DTS will also be explored. The aim is to model the requirements of the DTS and define the structural features and factors required for maximum interaction with the DTS. The information provided from this thesis will hopefully be applied in industry to drug discovery and development programmes in order to enhance oral absorption of drugs, either by modifying existing compounds, designing novel peptidomimetics to target the DTS, or converting poorly absorbed compounds into di-/tripeptide prodrugs.

CHAPTER 2

GENERAL MATERIALS AND METHODS

ABSTRACT

General materials and methods used routinely throughout experimental work are described within this chapter. Specific directions for individual experiments are given in the relevant chapters.

2.1 MATERIALS

Experimental studies were carried out at two locations, namely the Pharmaceutical and Sciences Institute at Aston University, Birmingham, UK, and the Metabolism and Pharmacokinetic (MAP) Department at Bristol-Myers Squibb, Princeton, New Jersey, USA. Therefore, two suppliers of materials will be listed, with the UK location appearing first, where appropriate.

2.1.1 CELL CULTURE REAGENTS AND MATERIALS

Caco-2 cells (passage 18/19), cell line-1, were purchased from the American Type Tissue Culture Collection (ATTC) (Rockville, MD, USA) and the European Collection of Animal Cell Cultures (ECACC) (Wiltshire, UK). Cultures (passage 95), cell line-2, used for the high-performance liquid chromatography (HPLC) studies were generously donated by Dr. Paul Nicklin of Ciba Pharmaceuticals (Horsham, UK).

Dulbecco's modified Eagle's medium (DMEM) and *N*-2-hydroxyethylpiperazine-*N'*-2-ethansulphonic acid (HEPES) buffer solution (1 *M*) were obtained from Gibco BRL, (Paisley, UK: Grand Island, NY, USA). Foetal bovine serum (FBS), L-glutamine (200 mM), non-essential amino acids (NEAA), penicillin, streptomycin and trypsin were all from Gibco BRL, (Paisley, UK), when in the UK. The bicinchoninic acid (BCA) protein assay reagent kit, Neubauer haemocytometer, and all other chemicals used while in the UK, unless otherwise stated were purchased from Sigma Chemical Company, (Poole, Dorset, UK). While in the USA, FBS was purchased from Hyclone Lab. Inc. (Logan, Utah, USA), ethylene diamine tetraacetic acid (EDTA), L-glutamine, penicillin, streptomycin and trypsin were obtained from JHR Bioscience (Lenexa, KS, USA). The BCA protein assay reagent kit was obtained from Pierce (Rockford, IL, USA). The Neubauer haemocytometer and all other chemicals used while in the USA, unless otherwise stated, were purchased from Sigma Chemical Company, (St. Louis, MO, USA). The culture flasks, 6-well, 24-well and 96-well plates were purchased from Falcon Labware distributed by Fahrenheit (Nottingham, UK) when in the UK, and from Costar (Cambridge, MA, USA) while in the USA.

FBS, L-glutamine, penicillin, trypsin and streptomycin were stored at -20°C and thawed prior to use in a water bath at 37°C. DMEM, HEPES 1 *M* buffer solution and NEAA were stored at 4°C. All other chemicals were stored at room temperature.

2.1.2 COMPETITION STUDIES

The dipeptide probe used throughout the project was glycyl-3, 4-[^{3}H]L-proline (Gly-[^{3}H]L-Pro (50 Ci mmol^{-1}) from Dupont New England Nuclear (NEN) Research Products (Boston, MA, USA). The imino-acid L-[2, 3, 4, 5-^{3}H]proline ([^{3}H]L-Pro) (100 Ci mmol^{-1}) was from Dupont NEN Research Products (Stevenage, Herts., UK: Boston, MA, USA).

N-tert-butoxy-carbonyl-L-prolinamide (*N*-Boc-L-prolinamide) and 1-methyl-2-pyrrolidineethanol were purchased from Aldrich Chemical Company Inc. (Milwaukee, WI, USA). *N*-Succinyl-L-proline and *N*-acetyl-L-proline were obtained from Sigma Chemical

Company (St. Louis, MO, USA). Gly-L-Pro-4 OH was from Bachem, (King of Prussia, PA, USA). *N*-Acetyl-L-proline amide was from Schweizerhall Inc. (South Plainfield, NJ, USA). *N*-Acetyl-L-hydroxyproline was from Fluka Chemical Corporation (Ronkonkoma, NY, USA). *N*-Boc-L-proline-t-butyl ester was from Lancaster (Windham, NH, USA). The dipeptides used throughout were all purchased from Sigma Chemical Company (Poole, Dorset, UK: St. Louis, MO, USA), with the exception of sarcosyl-L-proline (Sar-L-Pro) and L-Phe-L-Pro which were obtained from Schweizerhall Inc. (South Plainfield, NJ, USA) and Aldrich Chemical Company Inc. (Milwaukee, WI, USA) respectively. The following dipeptides were custom synthesised by Alta Bioscience (Birmingham, UK); D-alanyl-D-proline (D-Ala-D-Pro), L-alanyl-D-proline (L-Ala-D-Pro), D-lysyl-L-proline (D-Lys-L-Pro), L-lysyl-D-proline (L-Lys-D-Pro), D-lysyl-D-proline (D-Lys-D-Pro), L-norleucyl- L-proline (L-norLeu- L-Pro), L-ornithyl-L-proline (L-Orn-L-Pro) and 2 amino adipyl-L-proline (2 amino adipyl-L-Pro). The cephalosporins and penicillins were purchased from Sigma Chemical Company (Poole, Dorset, UK: St. Louis, MO, USA) and the carbapenems were a kind gift from Dr. P. A. Lambert, Aston University, Birmingham, UK. The ACE-inhibitors and the angiotensin-converting enzyme neutral endopeptidase (ACE-NEP) inhibitors were all provided by Bristol-Myers Squibb (Moreton, UK: Princeton, NJ, USA), with the exception of benazepril and benazeprilat which were kind gifts from Dr. R. Webb, Ciba (Summit, NJ, USA) and Roche (Welwyn Garden City, Hertfordshire, UK) for provision of the cilazapril. All SQ-29852 and glycyl-L-proline (Gly-L-Pro) analogues were from the in-house library at Bristol-Myers Squibb (Princeton, NJ, USA). All reagents for the ODN synthesis were obtained from Cruachem Limited (Glasgow, UK). The ODN-conjugates were a gift from Professor T. Brown, Oswell DNA Service, University of Edinburgh (Edinburgh, UK). [γ^{32}P]-dATP was purchased from ICN Biomedicals Ltd. (Thame, Oxon, UK). T4 polynucleotide kinase was purchased from Bioline (London, UK).

2.1.3 TRANSPORT STUDIES

Transwell inserts, with a polycarbonate membrane (3 μm pore size), were purchased from Costar (Cambridge, MA, USA). The 6-well companion plates to be used with the inserts were from also form Costar (Cambridge, MA, USA). The inserts were coated with rat tail collagen-type 1, which was purchased from Collaborative Research Inc. (Bedford, MA). D-[1-^{14}C]Mannitol (D-[^{14}C]mannitol) (56 mCi mmol^{-1}) which was used for assessment of the tight junction formation was purchased from Dupont NEN Research Products (Boston, MA, USA).

2.1.4 THIN-LAYER CHROMATOGRAPHY (TLC)

Propan-2-ol, formic acid and HPLC grade water were all purchased from Fisher Scientific UK, (Loughborough, Leicester, UK). Precoated plastic backed sheets of silica gel 60 F_{254} with a layer thickness of 0.2 mm were purchased from Merck Ltd. (Poole, Dorset, UK).

2.1.5 HPLC

2.1.5.1 GLY-[^{3}H]L-PRO DETECTION

A Millenium HPLC system with a dual-piston reciprocating pump from Waters Chromatography Division, Millipore Corporation (Milford, MA) was used. A Inertsil octadecyl silane-2 (ODS-2), 5 μm, reverse phase column (4.6 x 250 mm) was purchased from Waters Chromatography Division, Millipore Corporation (Milford, MA).

2.1.5.2 TRIPEPTIDE DETECTION

A Waters HPLC system with a dual-piston reciprocating pump from Waters (Watford, Herts., UK) was used to assay the samples by reversed-phase partition chromatography for each sample. A Hypersil 100 RP-18 endcapped column (5 μm silica spherical particles), 25 cm long, in-house packed column with an internal diameter of 4 mm was used. A guard column, 1 cm in length packed with octadecyl silane (ODS) coated stationary phase (1 - 2 mm internal diameter) was used to protect the column throughout the studies. The column, silica particles and ODS were all purchased from HPLC Technology (Macclesfield, Cheshire, UK). HPLC grade acetonitrile, diethylamine, orthophosphoric acid and HPLC grade water were all purchased from Fisher Scientific UK (Loughborough, Leicester, UK).

All chemicals used were of the highest purity and purchased from Sigma Chemical Company (Poole, Dorset, UK: St. Louis, MO, USA), unless otherwise stated. Chemicals and reagents were used as received unless stated, and all solvents were analytical grade.

2.2 METHODS

2.2.1 CELL CULTURE

All cell culture maintenance procedures were carried out under aseptic conditions in a Gelaire, biohazard level II, laminar flow cabinet from ICN (Thame, Oxfordshire, UK).

2.2.1.1 MEDIA

For the low passage number cells (18-40), the cell growth media (M1) comprised DMEM supplemented with 4.5 g l^{-1} glucose, 10 % v/v FBS, 1 % v/v NEAA, 1 % v/v L-glutamine (2 mM), 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 0.5 % HEPES buffer solution (5 μM). The high passage number cells were grown in two types of media, M2, a maintenance media, and M3, a plating media. M2 was DMEM supplemented with 4.5 g l^{-1} glucose, 10 % v/v FBS, 1 % v/v NEAA, and 1 % v/v L-glutamine. M3 was M2 with the addition of 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. The incubation medium (M4) for uptake and transport studies was Hanks balanced salt solution (HBSS) (9.8 g l^{-1}) containing 25 mM 2-[*N*-morpholino]ethanosulfonic acid (MES) adjusted to the

appropriate pH (5 - 6.5), or M5 containing HBSS (9.8 g l^{-1}) and 25 mM HEPES adjusted to the appropriate pH (6.5 - 7.4). 1*M* sodium hydroxide (NaOH) or hydrochloric acid (HCl) were used to adjust media to the appropriate pH values. M1, M2 and M3 were prepared under aseptic conditions, stored at 4°C and used within 14 d of preparation. M4 and M5 after preparation, were filtered using a 0.22 µm cellulose acetate bottle-top filter system purchased from Costar (Bucks, UK: Cambridge, MA, USA). A Na^+ free version of M4 and M5 were made up from the individual salts, omitting the sodium chloride (NaCL). All media was stored at 4°C and used within 14 d.

2.2.1.2 STOCK CULTURES

The cells were grown in plastic tissue-culture T-flasks, with a non-wetting 0.22 µm hydrophobic microporous membrane vent, and an area of 150 cm^2. The cultures were maintained in M1, or M2 (20 ml), depending on the cell line, which was replaced every 48 h. Cell line-1 was grown in an atmosphere of 5 % carbon dioxide (CO_2) (95 % air) and 90 % relative humidity, at 37°C, whereas, cell line-2 was grown in an atmosphere of 10 % CO_2 (90 % air) and 95 % relative humidity, at 37°C. The cultures were passaged by trypsinisation when confluent (monolayers appear as a sheet of paving stones with a regular spread of nuclei), every 3-4 d, and the flasks were seeded at approximately 4.7 x 10^4 cells cm^2. The following trypsinisation procedure was used: Aspirate the maintenance medium and wash the monolayers (1 x 10 ml x 5 min) with 0.25 % v/v phosphate-buffered saline (PBS)/EDTA at pH 7.2. Aspirate the PBS/EDTA, wash monolayers (1 x 5 ml x 35 secs) with 0.25 % v/v trypsin/EDTA (0.25 % v/v in PBS, pH 7.2), aspirate the trypsin/EDTA solution. Leave flasks at 37°C for 5-8 min, tap flask to dislodge cell sheet. Add 10 ml of M1/M2 (depending on the cell-line being used) to the flask to deactivate the remaining trypsin and wash over cells 10-15 times to achieve a single cell suspension. The cell suspension was then used to seed three flasks (3 ml $flask^{-1}$) to which 17 ml of M1/M2 was then added.

In order to check viable-cell density, a trypan-blue exclusion test was performed when seeding 6-, 24- and 96-well plates and inserts. 100 µl of trypan blue (4 mg ml^{-1}) was mixed with 400 µl of cell suspension following trypsinisation. 100 µl was then added to the counting chamber of an improved Neubauer haemocytometer, with depth 0.1 mM and area 1/400 mm^2. The cells in 5 large squares of the haemocytometer were counted under a light microscope. Viable cells appear with clear cytoplasm, whereas dead cells absorb the stain and appear blue. The cell density was calculated using the following equation:

EQUATION 2.1

$$\text{Cells ml}^{-1} = \frac{\text{number of cells counted}}{5} \times 10^4 \times 1.25 \text{ (dilution factor of trypan blue)}$$

Cell suspensions where the viable cells accounted for less than 96 % were discarded.

2.2.1.3 COMPETITION STUDIES

For competition studies, the cells (cell line-1) were used between passage number 25 to 40, and seeded onto 96-well tissue culture plates (area = 0.32 cm^2) at a density of 8 x 10^4 cell cm^2 (2.6 x 10^4 cells $well^{-1}$) or 24-well tissue culture plates (area = 2 cm^2) at a density of 8 x 10^4 cell cm^{-2} (1.6 x 10^5 cells $well^{-1}$). The plates were grown in an atmosphere of 5 % CO_2 (95 % air) and 90 % relative humidity, at 37°C. M1 (2 ml) was renewed every 48 h and the monolayers were used after 7 d of growth, unless otherwise stated.

2.2.1.4 TRANSPORT STUDIES

For transport studies, the cells (cell line-1) were used between passage number 25 to 35. The cell were seeded onto polycarbonate collagen coated inserts which were housed inside 6-well plates, with 2.6 ml of M1 in the basolateral compartment. The seeding density was 3.8 x 10^5 cells $insert^{-1}$ (8 x 10^4 cells cm^{2-1}) in a 1.5 ml volume. The cultures were then incubated at 37°C, in an atmosphere of 5 % CO_2 (95 % air) and 95 % relative humidity. M1 was renewed every 48 h and the inserts were used after 21 - 28 d post-seeding.

2.2.1.5 HPLC STUDIES

Cell line-2 (passage 95-110) was used for the tripeptide stability HPLC studies. Cells were trypsinised as previously described (see 2.2.1.2), except 10 ml of M3 was added to deactivate the trypsin and produce a single cell-suspension. The cells were seeded onto 6-well plates with 5 ml of cell suspension (2 x 10^5 cells ml^{-1}), giving a final seeding density of 1 x 10^6 cells $well^{-1}$. The plates were grown in an atmosphere of 10 % CO_2 (90 % air) and 90 % relative humidity, at 37°C. M3 was renewed every 48 h and the monolayers were used between 4 - 21 d, depending on the individual experiment.

2.2.2 ANALYTICAL METHODS

2.2.2.1 LIQUID SCINTILLATION COUNTING (LSC)

[^{3}H], [^{14}C] and [^{32}P] which are beta-emitting radionucleotides, were quantified using liquid scintillation spectrophotometry. Two methods were used depending on location;

1) LOCATION A

Sample (1 ml) + 10 ml Optiphase HiSafe III (LKB, UK),
Hewlett Packard Tricard 2000 CA liquid scintillation analyser,
10 min count-time.

2) LOCATION B

Sample (1 ml) + 15 ml Ecolite^{+}(ICN, CA, USA),
Hewlett Packard 2500 CA liquid scintillation analyser,
10 min count-time.

Counts per min (CPM) were converted to decays per min (DPM) by comparison to standard quench curves.

2.2.2.2 PROTEIN DETERMINATION

Protein content of the cell monolayers were determined using a BCA protein assay kit. The protein reduces copper (Cu) (II) to Cu (I) in a concentration-dependent manner, the BCA is a chromogenic reagent for Cu (I), producing a purple complex with a maximum absorbance at 562 nm, therefore the absorbance at 562 nm is proportional to protein concentration.

The following procedure was used: A 1 % Triton X-100 (TX-100) solution (in double distilled water), was used as the standard solution instead of water, due to it being used to remove the cell sheets from the plates. The protein determination reagent was produced by adding 1 part copper (II) sulphate pentahydrate 4 % solution to 50 parts BCA solution, both of which are provided within the kit. A protein standard solution (1 ml of 1 mg ml^{-1} bovine serum albumin) which was diluted with TX-100 to produce a range of concentrations (100 µg ml^{-1} - 1000 µg ml^{-1}) for the calibration curve, was also provided. 100 µl of the sample to be assayed (protein standards for calibration curve or unknowns) was mixed with 2 ml of the protein determination reagent and left at room temperature for 2 h. Absorbance at 562 nm was then read using a spectrophotometer. TX-100, which was used as the blank, had no effect on the absorbance when compared to water. The absorbance of the blank was subtracted from the remaining samples and a calibration curve was constructed by plotting the net absorbance vs. the concentration (µg ml^{-1}) of the protein standards at 562 nm. The calibration curve was then used to determine the amount of protein in the unknown samples.

2.2.3 COMPETITION STUDIES

The general method used for uptake/competition studies is given below: All solutions used throughout an experiment were preheated to 37°C, with the exception of the PBS-azide (stop-solution) containing 0.05 % w/v sodium azide which was used at 4°C, unless otherwise stated. The maintenance media (M1) was aspirated and the monolayers were washed with M4 at pH 6 (1 x 500 µl x 5 min) at 37°C, unless otherwise stated. The incubation solution (M4 at pH 6, unless otherwise stated) containing the required amount of the Gly-[^{3}H]L-Pro was then added to the well (1 x 250 µl x required time period) and incubated at 37°C unless otherwise stated, with agitation by an orbital shaker (50 cycles min^{-}

^{1}). Apicals were then removed, plates were transferred to the cold table (4°C) and washed with ice-cold PBS-azide solution (2 x 500 µl x 5 min). The monolayers were then harvested by solubilising with 1 ml of 1 % v/v TX-100 in doubled distilled water. Apical solutions, washings and the solubilised cells were collected made up to 1 ml with double distilled water, added to 10 ml of scintillation fluid and counted for ^{3}H or ^{32}P content by LSC (see 2.2.2.1).

2.2.4 TRANSPORT STUDIES

The general procedure for transport (insert) studies is outlined below: The maintenance media (M1) was aspirated and the inserts were washed [(1 x 1.5 ml apical with M4 at pH 6) + (1 x 2.6 ml basolateral with M5 at pH 7.4) x 15 min, unless otherwise stated] at 37°C. Washings were then aspirated and the inserts were transferred to fresh 6-well plates with 2.6 ml of M5 (pH 7.4) in each well. The experiment was initiated by adding the incubation media (1 x 1.5 ml of M4 at pH 6, unless otherwise stated) containing the appropriate probe (Gly-[^{3}H]L-Pro or [^{3}H]L-Pro). D-[^{14}C]mannitol (1 µM) was included in the apical phase to monitor the quality of the tight junctions between the cells. The plates were incubated at 37°C with agitation by an orbital shaker (50 cycles min^{-1}), with the inserts being transferred into fresh receiver solutions (basolaterals) after the required time period to maintain sink conditions. Samples were taken from each receiver (basolateral) well and the apical compartment was sampled at the end of the experiment. Samples were added to 15 ml Ecolite^{+} and counted for ^{3}H and ^{14}C content by LSC (see 2.2.2.1).

2.2.5 TLC STUDIES

A TLC method (Rajendran *et al.*, 1985a) using a mobile phase of propan-2-ol, formic acid and double distilled water in a ratio of 80:4:20, was used to identify [^{3}H]L-Pro. The stationary phase was pre-coated plastic-backed sheets of silica gel 60 F_{254} with a layer thickness of 0.2 mm. The chromatogram was run to 10 cm, and then dried rapidly at 70°C in an oven. The radiolabel distribution was determined by dividing the TLC plate into 0.5 cm horizontal strips, scraping off the silica coating from each area and adding it to 10 ml of HiSafe 3, with the ^{3}H content being determined by LSC.

2.2.6 HPLC STUDIES

2.2.6.1 GLY-[^{3}H]L-PRO DETECTION

The following protocol was used to detect Gly-[^{3}H]L-Pro. The mobile phase consisted of HPLC grade water with 0.1 % trifluoroacetic acid (TFA) which were mixed and filtered using a scintered glass filter with a pore-size of 0.45 µm. An isocratic run, with a flow-rate of 1 ml min^{-1} through the system was used. The mobile phase components were sparged throughout the experiment with helium gas (He), 15 ml min^{-1}, to expel oxygen from the system and the wavelength of detection was 210 nm.

2.2.6.2 TRIPEPTIDE DETECTION

The following HPLC protocol was used to identify the tripeptide phenylalanylglycylglycine (L-Phe-Gly-Gly) the breakdown products (L-Phe and L-Phe-Gly), by reversed-phase high-performance chromatography. The mobile phase consisted of HPLC grade water (94.9 %), acetonitrile (5 %) and diethylamine (0.1 %), which were mixed, filtered using a scintered glass filter with a pore-size of 0.45 μm, and adjusted to pH 3.5 with orthophosphoric acid (H_3PO_4). An isocratic run, with a flow-rate of 1 ml min^{-1} through the system, was used. The mobile phase components were sparged throughout the experiment with He gas, 15 ml min^{-1}, to expel oxygen from the system. The injection volume was 200 μl and the wavelength of detection was 255 nm. L-Trp (25 μg ml^{-1}), which had a rention time of 20 min, was used as the internal standard. L-Phe, L-Phe-Gly and L-Phe-Gly-Gly produced the following retention times; 9, 14, and 12 min respectively. Details of experiments whereby this HPLC method was used to analyse samples, are given in chapter 3 (section 3.2.6).

2.4 DATA ANALYSIS

The data sets are expressed as the mean ± standard deviation (S.D.) from at least three monolayers. Results for uptake studies are expressed as moles of the relevant compound being associated with the cell monolayer (nmol/pmol mg $protein^{-1}$ min^{-1}), or pmol/nmol min^{-1} $well^{-1}$, or % uptake of the total probe present. For competition studies, results are expressed as % inhibitions and IC_{50} ± S.D. (mM) (for IC_{50} derivation see section 3.3.2). For the ODN studies (Chapter 7) results are expressed as % inhibition of the control value (Gly-[^{3}H]L-Pro uptake into the cell monolayers, or [^{32}P]-S-rev associated with the cell monolayer).

For the transport studies, the results are expressed as the mean ± S.D. of amount transported, pmol h^{-1} $insert^{-1}$ or % inhibition of the control (Gly-L-Pro transport in the absence of any competitor).

For the TLC studies, results are expressed as % of total dpm on plates. The results for the HPLC studies are expressed as amount (mM) remaining per defined time period. Significance testing was performed on all results using an unpaired Student's t-test assuming equal variance (Gaussian population) or where the variance are significantly different from one another (Welch's test).

CHAPTER 3

GLY-[^{3}H]L-PRO UPTAKE INTO CACO-2 CELLS: DEVELOPMENT AND CHARACTERISATION OF A MODEL TO BE USED IN EVALUATING THE STRUCTURAL REQUIREMENTS FOR THE INTESTINAL DTS

ABSTRACT

The system established within this chapter has been designed and characterised for use in evaluating the structural requirements needed for recognition by the DTS. Gly-[^{3}H]L-Pro uptake into Caco-2 cells was found to be *via* an active carrier-mediated process having a K_m of 0.389 ± 0.002 mM, a V_{max} of 0.983 ± 0.043 nmol min^{-1} mg protein^{-1}, and a k_d of 0.245 ± 0.005 nmol min^{-1} mg protein^{-1} mM, accounting for 85 % of total uptake. Gly-[^{3}H]L-Pro uptake was found to be pH- and temperature-dependent, having an E_a of 46.67 kJ.mol^{-1}, confirming an active process. The cells were also shown to possess an active imino-acid carrier system, accounting for 56.41 ± 1.09 % of total uptake of L-[^{3}H]Pro, at 10 mM. Excess L-Pro (10 mM), which was included in the incubation solution in order to block active uptake of L-[^{3}H]Pro produced by hydrolysis of the probe, inhibited Gly-[^{3}H]L-Pro uptake by 19.05 ± 6.74 % and 6.68 ± 2.38 % (not significant), at UK and USA locations, respectively, indicating that clones differ in brush-border enzymatic expression, between laboratories. Thus 10 mM L-Pro was included in the apical solution of all future experiments. Gly-[^{3}H]L-Pro uptake was shown to be *via* the DTS and binding to ACE at the brush-border membrane was excluded. SQ-29852 (a specific probe for the DTS) inhibited uptake in a competitive manner. A range of amino acids tested failed to produce inhibition of uptake, with the exception of L-Trp. All the dipeptides tested produced some degree of inhibition, indicating that the present system is capable of ranking affinity for the DTS.

Gly-[^{3}H]L-Pro transport across Caco-2 cell monolayers was inhibited by cephradine, Gly-Sar, captopril and GABA. Transport was also shown to be reduced in the absence of Na^+ and inhibited in the presence of L-Pro.

L-Phe-Gly-Gly was hydrolysed at the brush-border membrane of the Caco-2 cells into L-Phe and the dipeptide Gly-Gly. Hydrolysis occurred in media that had been in contact with the cell monolayer, indicating that enzyme may be released into the extracellular environment or cell shedding may occur.

3.1 BACKGROUND

3.1.1. INTRODUCTION

There are several drugs, *e.g.* ACE-inhibitors and aminocephalosporins, that are absorbed to some extent by the intestinal DTS. However, their absorption mechanism were discovered after their selection as orally-active clinical candidates. An understanding of the structural requirements of the DTS may permit rational drug design to obtain compounds that are more efficiently orally absorbed *via* this system. The aim of this chapter was to design, and characterise an uptake model system using a stable probe, which would allow investigation of the structural features required for interaction with the DTS.

Many studies for drug absorption have used Caco-2 cells in a transport model, however, in the present work, an uptake model has been selected for the structural activity relationships studies for a number of reasons: Firstly, SQ-29852 uptake in Caco-2 monolayers has been shown to be an active process, however the basolateral efflux of the drug was found to be a passive process which provides the rate-limiting step [Nicklin *et al.*, 1996], thus indicating that an efflux transporter may be absent from, or much depleted in the basolateral membrane. Secondly, the cytoplasmic enzyme expression and composition in the Caco-2 cell line may differ from the *in vivo* situation and may therefore affect the intracellular metabolism and the chemical state in which a compound leaves the cell (efflux mechanisms). Thirdly, the Caco-2 cell model has been shown to under-estimate the predicted oral *in vivo* absorption of several actively absorbed drugs [Chong *et al.*, 1995], thus suggesting that the Caco-2 cells do not model the multi-component structure of the GI tract and that competing pathways may exist. Finally, the system has been designed as a potential indicator for recognition by the DTS, with the ability to screen a large number of dipeptide-like analogues. The uptake system offers a further advantage over the transport model in this regard; the time period required between seeding and experimentation is 7-9 d in our model, compared to 21-28 d for transport studies, thus making the system more cost and time effective.

3.1.2 GLY-[^{3}H]L-PRO ABSORPTION STUDIES

Gly-[^{3}H]L-Pro is a dipeptide which resembles the ACE-inhibitor structure and has a *C*-terminal Pro residue in common with these drugs. Studies in a variety of models have found Gly-[^{3}H]L-Pro uptake/transport to be an active process, *via* the DTS. Transport of Gly-L-Pro across the mucosal border of rabbit ileum suggested two saturable influx processes, with differing kinetic parameters, one with a V_{max} of 0.59 μmol cm^{-2} h^{-1} and an apparent affinity constant (K_t) of 0.93 mM and the other with a V_{max} of 4.03 μmol cm^{-2} h^{-1} and a K_t of 56.8 mM [Rubino *et al.*, 1971]. Gly-L-Pro influx was found to be Na^+-dependent, although in Na^+-free media, influx was greater than could be attributed to passive diffusion. Influx was inhibited by dipeptides except those which lacked a free NH_2-group in the α-position [Rubino *et al.*, 1971]. The extent of the Na^+-dependence of the

transporter was investigated 10 years later by Ganapathy *et al.*, who found that transport into rabbit intestinal and renal BBMV was active, Na^+-independent, and inhibited by various dipeptides in the presence of Na^+ and under Na^+-free conditions [Ganapathy *et al.*, 1981]. The suggestion of two transport systems was supported by findings from two independent research groups. Lane and colleagues studied Gly-L-Pro and L-Pro-Gly absorption in rats (*in vivo*) using a steady-state perfusion technique with an open-loop method [Lane *et al.*, 1975]. Gly-L-Pro was absorbed faster than L-Pro-Gly. L-Pro-Gly (40 mM) did not inhibit absorption of Gly-L-Pro (10 mM), whereas Gly-L-Pro (40 mM) inhibited absorption of the other dipeptide, suggesting that absorption of these two dipeptides may occur *via* different transport systems, with some overlap at high concentrations [Lane *et al.*, 1975]. Skopicki *et al.*, using rabbit renal BBMV also revealed the presence of two carriers; a lower affinity, higher capacity carrier (K_m = 1.3 x 10^{-2} M; V_{max} = 4.6 x 10^{-8} mol.mg^{-1}.min^{-1}) and a higher affinity lower capacity carrier (K_m = 2.7 x 10^{-7} M; V_{max} = 7.8 x 10^{-13} mol.mg^{-1}.min^{-2}). Both carriers also exhibit different inhibition profiles [Skopicki *et al.*, 1991]. Wootton found that Gly-L-Pro transport in rabbit intestinal BBMV was Na^+-independent and active, having the following kinetic parameters; K_m = 3.50 ± 0.36 mM, V_{max} = 43.5 ± 3.7 pmol mg^{-1} sec^{-1} and k_d = 1.94 ± 0.10 µl mg^{-1} sec^{-1}. Transport was inhibited by Gly-L-Phe and Gly-Gly, with K_i values of 1.93 ± 0.53 mM and 8.9 ± 1.5 mM, respectively [Wootton, 1986]. The model was later used [Wootton & Hazelwood, 1989] to characterise the relative affinities of a series of charged dipeptides. It was found that the dipeptide carrier of rabbit intestinal mucosal cells is tolerant of a single positive charge, but a negative or a double positive charge result in a loss of affinity. Gly-L-Pro transport in rabbit intestinal and renal BBMV has also been found to result in the net transfer of a positive charge [Ganapathy & Leibach, 1983], indicating that the DTS H^+/cotransport system is utilised. Transport across mouse intestinal BBMV has also been shown to be *via* a carrier-mediated Na^+-independent process, which is inhibited by dipeptides containing L-amino acids but not by dipeptides containing D-amino acids [Rajendran *et al.*, 1985b]. Human intestinal BBMV transports Gly-L-Pro *via* an active Na^+-independent process with a K_m of 4.1 ± 0.5 mM and a V_{max} of 1.53 ± 0.07 nmol mg protein^{-1} 0.5 min^{-1}. Transport has been shown to be inhibited by both di- and tripeptides [Rajendran *et al.*, 1985a]. Recently, the existence of a peptide transporter in the ruminant small intestine has also been demonstrated [Backwell *et al.*, 1995]. In conclusion, it appears that Gly-L-Pro is absorbed *via* the DTS in a number of animal models, by a Na^+-independent process (Na^+ seems to play an indirect role in some studies). One study using Caco-2 cells looking at uptake of Gly-L-Pro, is available within the literature [Eddy *et al.*, 1993]. It states that Scatchard plots at both pH 6 and pH 7.4 indicate that two binding sites exist, a conclusion which is supported by the fact that inhibition of uptake was seen with a range of dipeptides and cephalosporins but not with L-glutamyl-L-glutamate (L-Glu-L-Glu) [Eddy *et al.*, 1993]. However, biphasic plots may well be attributable to uptake of free amino acid residues released by hydrolysis of the probe at the brush border membrane. Therefore, the total kinetic curve represents amino acid and dipeptide uptake

giving the impression of two binding sites. Suppression of the amino acid uptake has been shown to produce linear plots [Matthews, 1987]. Therefore, caution must be taking when interpreting results claiming to support multiple transport systems.

3.1.3 PEPTIDE HYDROLYSIS

Proteins once ingested, are subject to enzymatic breakdown at several sites along the GI tract (*e.g.* luminally, at the brush border, and intracellularly). The enzymatic barrier is composed of exopeptidases which attack peptides and proteins at their *N*- and *C*-termini, and endopeptidases which cleave at internal peptide bonds [Lee & Yamamoto, 1990]. The first site of protein breakdown occurs in the stomach, were pepsins which are active at pH 2-3, begin to digest the proteins. The duodenum secretes pancreatic proteases (*e.g.* trypsin, α-chymotrypsin, elastase and carboxypeptidase A) which cleave peptide bonds further. Trypsin cleaves bonds near basic amino acids (*e.g.* L-arginine {L-Arg} and Lys), while α-chymotrypsin works near hydrophobic amino acids (*e.g.* Leu, Met, Phe, Trp and Tyr). Elastase cleaves peptide bonds of amino acids, such as Gly, Ser and Val, with smaller unbranched, non-aromatic side chains [Naughton & Sanger, 1961], while carboxypeptidase A works on amino acids that have a free carboxyl group and a *C*-terminal L-amino acid [Neurath & Schwert, 1950]. Small peptides are resistant to pancreatic proteases, with the majority of hydrolysis occurring at the brush-border or intracellularly [Silk *et al.*, 1976] with luminal hydrolysis, if it occurs, accounting for only 5 -20 % of total degradation [Adibi, 1971: Crampton *et al.*, 1973]. The brush-border proteases consist of aminopeptidase A and N, diaminopeptidase IV, endopeptidase 24.11, ACE, Gly-Leu peptidase, and L-aspartyl-L-lysine (L-Asp-L-Lys) peptidase [Lee & Yamamoto, 1990], which are anchored to the apical membrane with the active site in the extracellular environment [Feracci *et al.*, 1982: Hussain, 1986]. Overall degradation of small peptides depends on two factors, the size and amino acid composition. It has been suggested that some dipeptides are hydrolysed by brush-border peptidases and absorbed *via* amino acid carriers, whereas hydrolysis-resistant peptides are transporpted into the cell *via* the DTS and hydrolysed by cytoplasmic peptidases, which have a preference for dipeptides [Catnach & Fairclough, 1994]. The site of hydrolysis is dependent upon the resistance of a given peptide to the peptidases at the brush-border membrane. The cytoplasmic and brush-border peptidases are complementary to each other and small peptides that are poor substrates for the brush-border membrane peptidases are excellent substrates for the DTS and cytoplasmic peptidases. Brush-border enzymes are capable of hydrolysing peptides up to 10 amino acid residues [Matthews & Payne, 1980]. However, they prefer tri-and tetrapeptides to dipeptides and particularly peptides with a lipophilic side chain at the *N*-terminus residue [Matthews & Payne, 1980]. Naturally occurring peptides, which have been found to be hydrolysis-resistant include carnosine, and peptides of Gly and L-Pro [Matthews, 1987]. For example, the dipeptide Gly-L-Pro was found to be 60,000 times less susceptible to hydrolysis by rabbit renal BBMV than L-alanyl-glycine (L-Ala-Gly) [Ganapathy *et al.*, 1980].

3.1.4 PEPTIDASE ACTIVITY IN CACO-2 CELL MONOLAYERS

Caco-2 cell monolayers have been shown to express the following eight membrane peptidases; ACE, aminopeptidase-N, aminopeptidase-P, aminopeptidase-W, dipeptidyl peptidase IV, endopeptidase-24.11, membrane dipeptidase (MDP) and γ-glutamyl transpeptidase [Howell *et al.*, 1992]. Activity of all enzymes were found to increase after confluency, however the activity profiles differed. MDP and aminopeptidase-P, which have been shown to play a role in the release of the *N*-terminal amino acid from peptides with a penultimate propyl residue [Hooper *et al.*, 1990] appear to be anchored to the cell membrane by a covalently attached glycosyl-phosphatidylinositol moiety [Howell *et al.*, 1992]. Endopeptidase-24.11 is a 94-kDa membrane-bound Zn-metalloprotease located in the plasma membrane of many tissues [Kenny, 1986], and hydrolyses peptide bonds involving the amino group of hydrophobic residues, preferring small peptides to proteins [Matsas *et al.*, 1984]. Endopeptidase-24.11 has been found to have a polarised cell surface expression at the apical membrane of Caco-2 cells grown on filters, with similar enzymatic and biochemical properties to that found in rabbit kidney [Jalal *et al.*, 1992]. ACE, aminopeptidase-P, aminopeptidase-W and endopeptidase-24.11 all show mosaic (heterogeneous) expression in Caco-2 cells [Howell *et al.*, 1993]. However, homogeneous expression was observed after permeabilisation, indicating that storage pools for the enzymes in question, are located within the cells [Howell *et al.*, 1993]. These results illustrate that, within a population, different phenotypes exist with respect to enzymatic expression. Possible explanations for this are the fact that apical transport of these enzymes may differ, the enzymes may be recycled at different rates from the surface to the interior or enzyme expression may be modulated by cell to cell interactions or by local factors [Howell *et al.*, 1993].

3.2 MATERIALS AND METHODS

3.2.1 MATERIALS

Details of the suppliers of all materials used throughout this chapter have been described previously (section 2.1- 2.1.3).

3.2.2 METHODS

3.2.2.1 MEDIA

Details of cell growth media, M1, M2 and M3; and incubation media, M4 and M5 used throughout this chapter have been described previously (section 2.2.1.1). Na^+-free versions of M4 and M5 were made omitting the NaCl from the salt mixture.

3.2.2.2 CELL CULTURE

Cell line-1 described previously (section 2.1.1), was used between passage number 25 to 40. The cells were seeded onto 24-well plates (2 cm^2) at a density of 1.6 x 10^5 cells $well^{-1}$ or onto 96-well plates (0.32 cm^2) at a density of 2.6 x 10^4 cells $well^{-1}$, unless otherwise stated, and grown in an atmosphere of 5 % CO_2 (95 % air) and 90 % relative humidity, at 37°C, as previously described (section 2.2.1.3). M1/M2 (2 ml) were renewed every 48 h and the monolayers were used after 7 d growth, unless otherwise stated.

3.2.2.3 GENERAL EXPERIMENTAL CONDITIONS FOR UPTAKE STUDIES

The following general procedure and conditions are used for all studies unless otherwise stated: All solutions were preincubated at 37°C prior to experimentation, with the exception of PBS-azide which is kept at 4°C. The maintenance media was aspirated and the monolayers washed with M4 at pH 6 (1 x 500 µl x required time period {5 - 15 min}) at 37°C. The incubation solution (M4 at pH 6) containing the required amount of probe (Gly-[^{3}H]L-Pro or [^{3}H]L-Pro), was added to the well (1 x 250 µl x required time period {0.5 - 60 min}) and incubated at 37°C, with agitation by an orbital shaker (50 cycles min^{-1}). Apical solutions were collected, and the plates transferred to the cold table (4°C) and washed with ice-cold PBS-azide solution (2 x 500 µl x 5 min). The monolayers were then harvested by solubilising with 1 ml of 1 % v/v TX-100 in double distilled water. Apical solutions, washings and solubilised cells were collected, made up to 1 ml with double distilled water, added to 10 ml of HiSafe 3 (UK) or 15 ml Ecolite$^+$ (USA) and counted for ^{3}H-content by LSC. All conditions are as described above, unless otherwise stated.

3.2.2.4 PROTEIN DETERMINATION

The protein content of cell monolayers were determined by using a BCA assay as previously described (section 2.2.2.2). The accuracy of the assay was investigated by determining the protein concentration of a range of cell suspensions containing a known number of cells (1.04 x 10^5 - 6.3 x 10^5 cells ml^{-1}).

3.2.3 GLY-[^{3}H]L-PRO UPTAKE INTO CACO-2 CELLS MONOLAYERS: DEVELOPMENT OF THE SYSTEM

3.2.3.1 ASSESSMENT OF [^{3}H]L-PRO PURITY

The purity of the [^{3}H]L-Pro was assessed prior to experimentation, using the TLC method as described previously (section 2.2.5). The loading volume of [^{3}H]L-Pro on the plate was 2 µl.

3.2.3.2 ASSESSMENT OF GLY-[^{3}H]L-PRO PURITY

The purity of Gly-[^{3}H]L-Pro was assessed prior to experimentation by Sandra Dando, (Bristol-Myers Squibb, Princeton, USA) using the HPLC method as described

previously (section 2.2.6.1). 5 μCi of Gly-[^{3}H]L-Pro was co-eluted with 10 μg of unlabelled Gly-L-Pro. [^{3}H]L-Pro was also co-injected as a standard.

3.2.3.3 THE EFFECT OF EXCESS L-PRO ON THE UPTAKE OF [^{3}H]L-PRO AND GLY-[^{3}H]L-PRO INTO CACO-2 CELL MONOLAYERS

The cells were seeded onto 24-well plates as previously described (section 2.2.1.3), and used at 7 d post-seeding. The incubation solution contained 100 nM [^{3}H]L-Pro in M4 at pH 6, and the incubation period was for a duration of 3 min. The experiment was repeated with the addition of 10 mM L-Pro to the incubation solution. The effect of 10 mM L-Pro on the uptake of Gly-[^{3}H]L-Pro was investigated by replacing the 100 nM [^{3}H]L-Pro with 80 nM Gly-[^{3}H]L-Pro and 50 nM unlabelled Gly-L-Pro, in the above procedure. The procedure was carried out while in the UK and USA, and results were compared.

3.2.3.4 KINETIC PROFILE OF GLY-[^{3}H]L-PRO UPTAKE INTO CACO-2 CELL MONOLAYERS

3.2.3.4.1 Uptake over a 60 min period

A preliminary experiment was carried out using Caco-2 cells (cell line-1) seeded onto 96-well plates as previously described (section 2.2.1.3), used at 7 d post-seeding. The incubation solution consisted of 10 mM L-Pro, 50 nM unlabelled Gly-L-Pro and 40 nM of Gly-[^{3}H]L-Pro, in M4 at pH 6. Monolayers were washed, (1 x 200 μl x 5 min) at 37°C with M4 at pH 6, and then incubated with the incubation solution (1 x 50 μl x required time period {1 - 60 min}) at 37°C with agitation. Uptake was investigated at the following incubation time points; 1, 2, 4, 5, 10, 30, and 60 min. After incubation, the plates were transferred to the cold table (4°C) and monolayers were washed with ice-cold PBS-azide (2 x 200 μl x 5 min) at 4°C. Cells were harvested with 100 μl of 1 % v/v TX-100 in double distilled water. All solutions, apicals, washings and solubilised cells were collected and added to scintillation cocktail and counted for ^{3}H content by LSC.

3.2.3.4.2 Uptake over a 30 min period

The above experiment (*3.2.3.4.1*) was repeated using cells seeded onto 24-well plates as previously described (section 2.2.1.3), used at 7 d post-seeding. The incubation solution consisted of 10 mM L-Pro, 50 nM unlabelled Gly-L-Pro and 40 nM Gly-[^{3}H]L-Pro in M4 at pH 6. Uptake was investigated a the following time points; 0.5, 1, 1.5, 2, 3, 5, 10, 12.5, 15, 20, 20, 25 and 30 min.

3.2.3.5 SPECIFIC UPTAKE OF GLY-[^{3}H]L-PRO INTO CACO-2 CELL MONOLAYERS

The specific uptake of Gly-[^{3}H]L-Pro by ageing Caco-2 cells was determined by measuring uptake of Gly-[^{3}H]L-Pro at a low (130 nM) and high (5 mM) concentration over a 7 d period. The cells were seeded onto 24-well plates, as previously described (section

2.2.1.3), and used between 4-11 d post-seeding. The incubation solution contained 10 mM L-Pro, 80 nM Gly-[^{3}H]L-Pro and 50 nM or 5 mM of unlabelled Gly-L-Pro in M4 at pH 6, and the incubation period was 3 min. The % specific uptake was calculated by subtracting the % non-specific uptake, which corresponds to uptake in the presence of excess unlabelled Gly-L-Pro (5 mM), from the total uptake (130 nM). For calculation see appendix 2.

3.2.3.6 THE CONCENTRATION-DEPENDENCY OF GLY-[^{3}H]L-PRO UPTAKE

The cells were seeded onto 24-well plates as previously described (section 2.2.1.3), and used 7 d post-seeding. The incubation solution contained 10 mM L-Pro and 50 nM Gly-[^{3}H]L-Pro in M4 at pH 6, in the presence of a range of increasing concentrations of unlabelled Gly-L-Pro; 0 (control), 0.001 - 10 mM. The incubation period was 3 min.

3.2.3.7 THE EFFECT OF DIMETHYL SULPHOXIDE ON GLY-[^{3}H]L-PRO UPTAKE

The cells were seeded onto 24-well plates as previously described (section 2.2.1.3), and used 7 d post-seeding. Dimethyl sulphoxide (DMSO) was added to the incubation solution as a cosolvent to assist in dissolving poorly soluble competitors that were to be investigated in future experiments. The incubation solution contained 10 mM L-Pro, 50 nM Gly-[^{3}H]L-Pro and 0 (control), 1, 2, or 5 % DMSO, in M4 at pH 6, and the incubation period was 3 min. The experiment was repeated with the addition of 5 mM unlabelled Gly-L-Pro to the incubation solution.

3.2.3.8 THE TEMPERATURE-DEPENDENCY OF GLY-[^{3}H]L-PRO UPTAKE

The cells were seeded onto 24-well plates as previously described (section 2.2.1.3), and used 7 d post-seeding. The incubation solution contained 10 mM L-Pro and 50 nM Gly-[^{3}H]L-Pro in M4, at pH 6. Uptake was investigated at the following temperatures; 4, 19, 25.5, 30.5, and 37°C. All solutions were preincubated for 30 min at the required temperature. The cells were washed (1 x 500 µl x 30 min) with M4 at the appropriate temperature, and incubated with the experimental solutions at the appropriate temperature.

3.2.3.9 THE pH-DEPENDENCY OF GLY-[^{3}H]L-PRO UPTAKE

The cells were seeded onto 24-well plates as previously described (section 2.2.1.3), and used 7 d post-seeding. Uptake was investigated at the following pHs; 5, 5.5, 6, 6.5, 7 and pH 7.5. The incubation solution contained 10 mM L-Pro and 50 nM Gly-[^{3}H]L-Pro. M4 was used for the solutions at pH 5, 5.5, 6, and 6.5, and M5 was used for the solutions at pH 7 and 7.5. NaOH and HCl (1 *M*) were used to adjust the pH of solutions where required. The monolayers were washed (1 x 500 µl x 30 min) and then incubated (1 x 250 µl x 3 min) with M4 or M5 at the appropriate pH at 37°C.

3.2.3.10 THE EFFECT OF THE PRE-INCUBATION WASHING PROTOCOL ON GLY-[^{3}H]L-PRO UPTAKE

The cells were seeded onto 24-well plates as previously described (section 2.2.1.3), and used 7 d post-seeding. The incubation solution contained 10 mM L-Pro and 50 nM Gly-[^{3}H]L-Pro in M4 (containing 2 % DMSO) at pH 6. The preincubation washing procedures were as follows; A) 1 x 500 µl x 5 min, and B) 1 x 500 µl x 15 min.

3.2.3.11 THE EFFECT OF THE POST-INCUBATION WASHING PROTOCOL ON GLY-[^{3}H]L-PRO UPTAKE

The cells were seeded onto 24-well plates as previously described (section 2.2.1.3), and used 7 d post-seeding. The incubation solution contained 10 mM L-Pro and 50 nM Gly-[^{3}H]L-Pro in M4 (containing 2 % DMSO) at pH 6. The post-incubation washing procedures were as follows; A) 1 x 500 µl x 5 min, B) 2 x 500 µl x 5 min, C) 3 x 500 µl x 5 min and D) 4 x 500 µl x 5 min. Each wash was counted separately for ^{3}H content.

3.2.4 SPECIFICITY OF GLY-[^{3}H]L-PRO UPTAKE INTO CACO-2 CELL MONOLAYERS

3.2.4.1 EXPERIMENTAL CONDITIONS

The following conditions and general methods (section 3.2.2.3) were used throughout the specificity studies unless otherwise stated; The cells were seeded onto 24-well plates as previously described (section 2.2.1.3), and used 7-9 d post-seeding. The incubation solution consisted of 50 nM Gly-[^{3}H]L-Pro and 10 mM L-Pro in M4 containing 2 % DMSO, at pH 6. The incubation period was 3 min.

3.2.4.2 IS GLY-[^{3}H]L-PRO BINDING TO ACE RATHER THAN BEING A SUBSTRATE FOR THE DTS?

To evaluate whether Gly-[^{3}H]L-Pro was binding to ACE rather than being a substrate for the DTS, uptake of Gly-[^{3}H]L-Pro, described in section 3.2.4.1, was investigated with or without 1 µM fosinoprilat.

3.2.4.3 THE EFFECT OF THE ACE-INHIBITOR, SQ 29852, ON THE UPTAKE OF GLY-[^{3}H]L-PRO

3.2.4.3.1 Reproducibility of the system

SQ-29852 was selected as a potential competitor due to being a substrate for the DTS [Marino *et al.*, 1996: Nicklin *et al.*, 1996]. Uptake in the presence of 1 mM SQ-29852 on a number of experimental occasions was investigated in order to check the

reproducibility of the system. Inhibition (%) and IC_{50} values were compared between experimental occasions and cells with increasing passage numbers.

3.2.4.3.2 Binding mechanism of SQ-29852

The binding mechanism of SQ-29852 to the DTS was investigated. Gly-[^{3}H]L-Pro uptake at 0.13, 0.4, 3.6 and 49.6 mM were measured in the presence of the following concentrations of SQ-29852; 0 (control), 1 and 3 mM.

3.2.4.4 SPECIFICITY OF GLY-[^{3}H]L-PRO UPTAKE *VIA* THE DTS

The effect on Gly-[^{3}H]L-Pro uptake, by a series of compounds that are transported *via* other transport systems in Caco-2 cells, and a metabolic inhibitor, were investigated by co-administering them with the incubation solution, at a concentration of 1 mM. The following compounds were studied; bestatin, glutathione, glycyl-L-prolyl-glycylglycine (Gly-L-Pro-Gly-Gly), sodium azide (NaN_3), taurocholic acid (bile acid) and TRH.

3.2.4.5 THE EFFECT OF VARIOUS AMINO ACIDS AND DIPEPTIDES ON THE UPTAKE OF GLY-[^{3}H]L-PRO INTO CACO-2 CELL MONOLAYERS

The effect of various amino acids and dipeptides on Gly-[^{3}H]L-Pro uptake into Caco-2 cells was investigated by co-administering them with the incubation solution at a concentration of 1 mM. The following compounds were studied; L-Arg, Gly, L-Met, L-Phe, sarcosine (Sar), L-Ser, L-Trp, L-Tyr, L-alanyl-L-valine (L-Ala-L-Val), L-aspartyl-L-aspartate (L-Asp-L-Asp), carnosine (β-Ala-L-His), glycl-DL-methionine (Gly-DL-Met), glycyl-DL-phenylalanine (Gly-DL-Phe), Gly-Sar, glycyl-L-tryptophan (Gly-L-Trp), Gly-L-Tyr, L-Glu-L-Glu, L-Phe-Gly, L-Phe-L-Pro, L-prolyl-L-phenylalanine (L-Pro-L-Phe) and Sar-L-Pro.

3.2.5 GLY-[^{3}H]L-PRO TRANSPORT ACROSS CACO-2 CELL MONOLAYERS

The Gly-[^{3}H]L-pro transport studies were carried out in colaboration with Sandra Dando, Bristol-Myers Squibb, Princeton, USA.

3.2.5.1 GLY-[^{3}H]L-PRO TRANSPORT ACROSS CACO-2 CELL MONOLAYERS IN THE PRESENCE OF VARIOUS COMPETITORS

The cells were seeded onto polycarbonate inserts as previously described (section 2.2.1.4) and used after 24 d post-seeding. The general method for transport experiments has been described previously (section 2.2.4). The apical (incubation) solution contained 50 nM unlabelled Gly-L-Pro and 8 nM Gly-[^{3}H]L-Pro in M4, at pH 6. The following compounds were co-administered with the apical (incubation) solutions: captopril (10 mM), cephradine (10 mM), GABA (10 mM) and Gly-Sar (10 mM). In order to ascertain whether the probe was transported against a concentration gradient Gly-L-Pro (10 mM) was also included in the basolateral solution (pH 7.4) on one occasion. Gly-[^{3}H]L-Pro flux across the monolayers were monitored after 30 and 60 min.

3.2.5.2 THE EFFECT OF L-PRO ON THE TRANSPORT OF [^{3}H]L-PRO AND GLY-[^{3}H]L-PRO ACROSS CACO-2 CELL MONOLAYERS

The cells were seeded onto polycarbonate inserts as previously described (section 2.2.1.4) and used after 21 d post-seeding. Transport of [^{3}H]L-Pro and Gly-[^{3}H]L-Pro in the presence of excess L-Pro was compared. The following apical solutions were tested in the presence of 10 mM L-Pro;

A) 50 nM unlabelled L-Pro and 4 nM [^{3}H]L-Pro,

B) 50 nM unlabelled Gly-L-Pro and 4 nM Gly-[^{3}H]L-Pro.

[^{3}H]L-Pro and Gly-[^{3}H]L-Pro flux across the monolayers were monitored after 30 and 60 min.

3.2.5.3 THE EFFECT OF SODIUM ON THE TRANSPORT OF [^{3}H]L-PRO AND GLY-[^{3}H]L-PRO ACROSS CACO-2 CELL MONOLAYERS

The cells were seeded onto polycarbonate inserts as previously described (section 2.2.1.4) and used after 21 d post-seeding. The following apical solutions were tested in both M4 at pH 6, and Na^+-free M4 at pH 6;

A) 50 nM unlabelled L-Pro and 4 nM [^{3}H]L-Pro,

B) 50 nM unlabelled Gly-L-Pro and 4 nM Gly-[^{3}H]L-Pro.

The M5 at pH 7.4 used for the receiver phase (basolateral) was also Na^+-free. [^{3}H]L-Pro and Gly-[^{3}H]L-Pro flux across the monolayers were monitored after 30 and 60 min.

3.2.5.4 THE EFFECT OF THE ACE-INHIBITORS CAPTOPRIL AND SQ-29852 ON GLY-[^{3}H]L-PRO TRANSPORT ACROSS CACO-2 CELLS MONOLAYERS IN THE PRESENCE OF EXCESS L-PRO

The cells were seeded onto polycarbonate inserts as previously described (section 2.2.1.4) and used after 24 d post-seeding. The apical (incubation) solutions contained 4 nM Gly-[^{3}H]L-Pro, 50 nM unlabelled Gly-L-Pro and 10 mM L-Pro in M4, at pH 6, in the presence of one of the following compounds: L-captopril (1 mM), D-captopril (1 mM), SQ-29852 (1 mM) and SQ-29852 (10 mM). Gly-[^{3}H]L-Pro flux across the monolayers were monitored after 30 and 60 min.

3.2.6 TRIPEPTIDE STABILITY IN THE PRESENCE OF CACO-2 CELL MONOLAYERS

3.2.6.1 L-PHE-GLY-GLY STABILITY WHEN INCUBATED WITH CACO-2 CELL MONOLAYERS

The cells (cell line-2, passage 95 - 110), were seeded onto 6-well plates as previously described (section 2.2.1.5) and used after 7 d post-seeding. All solutions were preincubated at 37°C, prior to use. The monolayers were washed (1 x 2 ml x 30 min) at 37°C with M5, at pH 7.4. The monolayers were incubated with (1 x 2 ml) 1 mM L-Phe-Gly-Gly for the

following time points; 5, 10, 30, 60, 90, 120 and 240 min. Apicals were then removed and placed on ice, to stop/slow any enzymatic activity. Samples were assessed for L-Phe-Gly-Gly and L-Phe content using the HPLC method previously described (section 2.2.6.2).

3.2.6.2 THE EFFECT OF CELL AGE ON THE BREAKDOWN OF L-PHE-GLY-GLY WHEN INCUBATED WITH CACO-2 CELL MONOLAYERS

The above experiment (section 3.2.6.1) was repeated using Caco-2 cell monolayers after 4, 7, 10 and 21 d post-seeding. Stability was monitored at the following time points; 5, 15, 30, 60, 120, 240 and 360 min.

3.2.6.3 [^{3}H]L-PHE UPTAKE INTO CACO-2 CELL MONOLAYERS

The cells (cell line-2), were seeded onto 6-well plates as previously described (section 2.2.1.5) and used after 7 d post-seeding. Monolayers were washed (1 x 2 ml x 30 min) at 37°C with M5, at pH 7.4, and then incubated with 10.8 nM [^{3}H]L-Phe and 1 mM unlabelled L-Phe in M5 at pH 7.4. Uptake was investigated after the following time; 5, 10, 30, 60, 90, 120, 240 and 360 min. Apical solutions were removed, and the plates were transferred to the cold table (4°C) and washed with ice-cold PBS-azide solution (2 x 1 ml x 5 min) at 4°C. The monolayers were harvested by solubilising with 2 ml of 1 % v/v TX-100 in double distilled water. Apical solutions, washing and solubilised cells were added to 10 ml HiSafe 3 and counted for ^{3}H content by LSC.

3.2.6.4 ARE THE ENZYMES THAT BREAKDOWN L-PHE-GLY-GLY MEMBRANE BOUND OR RELEASED FROM THE CELL?

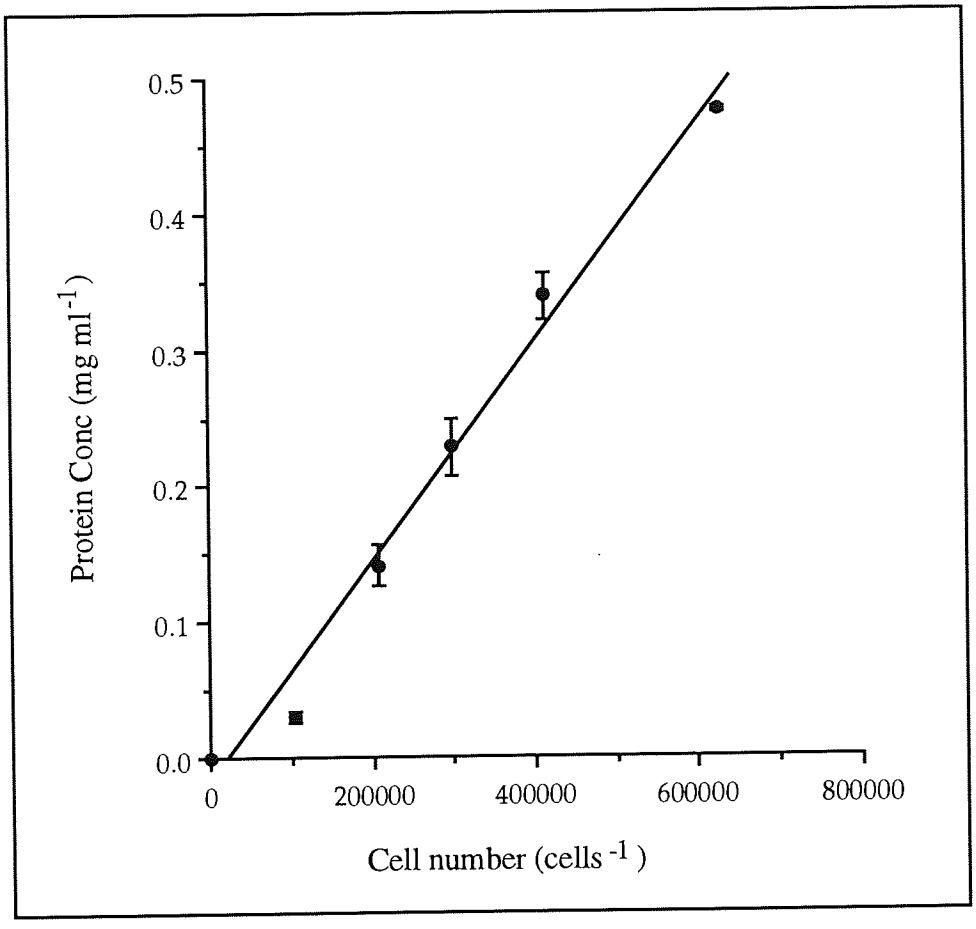
To assess whether the enzymes that cause degradation of the tripeptide are membrane-bound or released from the cell into the surrounding environment (apical solution), the following experiment was carried out. The cells (cell line-2), were seeded onto 6-well plates as previously described (section 2.2.1.5) and used after 7 d post-seeding. The monolayers were washed (1 x 2 ml x 30 min) at 37°C, with M5 at pH 7.4. The monolayers were then incubated (1 x 2 ml) with M5 at pH 7.4, at the following time points; 10, 30 and 60 min. The apicals were removed and added to 2 ml of 1 mM L-Phe -Gly-Gly solution, giving a final tripeptide concentration of 0.5 mM. The solutions were incubated at 37°C for 60 min, and then placed on ice, in order to slow/stop enzymatic degradation. The samples were then assessed for L-Phe-Gly-Gly and L-Phe content using the HPLC method previously described (section 2.2.6.2).

3.3 RESULTS AND DISCUSSION

In order to correct for differences in cell density of monolayers, a BCA protein assay was carried out on each experimental day. This also allowed for results to be expressed as mg protein^{-1}, where appropriate. The protein concentration of the monolayers grown on 24-well plates ranged from 300 g to 550 μg well^{-1}, depending on the age of the monolayers (the older the monolayer the higher the protein concentration). Figure 3.1 illustrates that the cell density of a suspension is proportional to the protein concentration determined from the BCA assay (coefficient of line $r^2 = 0.986$).

FIGURE 3.1 *Protein concentration of various cell suspensions*

A range of cell suspension were produced by diluting the stock solution (6.3 x 10^5 cells ml^{-1}) and protein content was determined using the BCA assay method.



3.3.1 GLY-[^{3}H]L-PRO UPTAKE INTO CACO-2 CELL MONOLAYERS: DEVELOPMENT OF THE SYSTEM

[^{3}H]L-Pro was assessed for contamination by radio-TLC (Figure 3.2). The [^{3}H]L-Pro produced one peak, accounting for 93.4 % of the total dpms, and having an R_f value of 0.3. Gly-[^{3}H]L-Pro was assessed for contamination by HPLC. One peak (Figure 3.3 A), with a retention time of 9.29 min was visible, accounting for 84.19 % of total cpms. The areas between 2.40-8.26 min, and 17.20-21.18 min accounted for 9.99 and 5.82 % respectively. [^{3}H]L-Pro (Figure 3.3 B) was co-injected in order to check that the peak at 9.29 min is due to Gly-[^{3}H]L-Pro and not [^{3}H]L-Pro resulting for degradation. Two separate peaks were produced, with [^{3}H]L-Pro being eluted earlier than the Gly-[^{3}H]L-Pro, and having a retention time of 4.18 min. Unfortunately, this method was not feasible for assessing breakdown of the probe in the presence of Caco-2 cells, as the peaks for [^{3}H]L-Pro and Gly-[^{3}H]L-Pro overlapped under these conditions.

FIGURE 3.2 *Identification and purity assessment of [^{3}H]L-Pro*

Radio-TLC chromatogram showing tritium-distribution of [^{3}H]L-Pro.

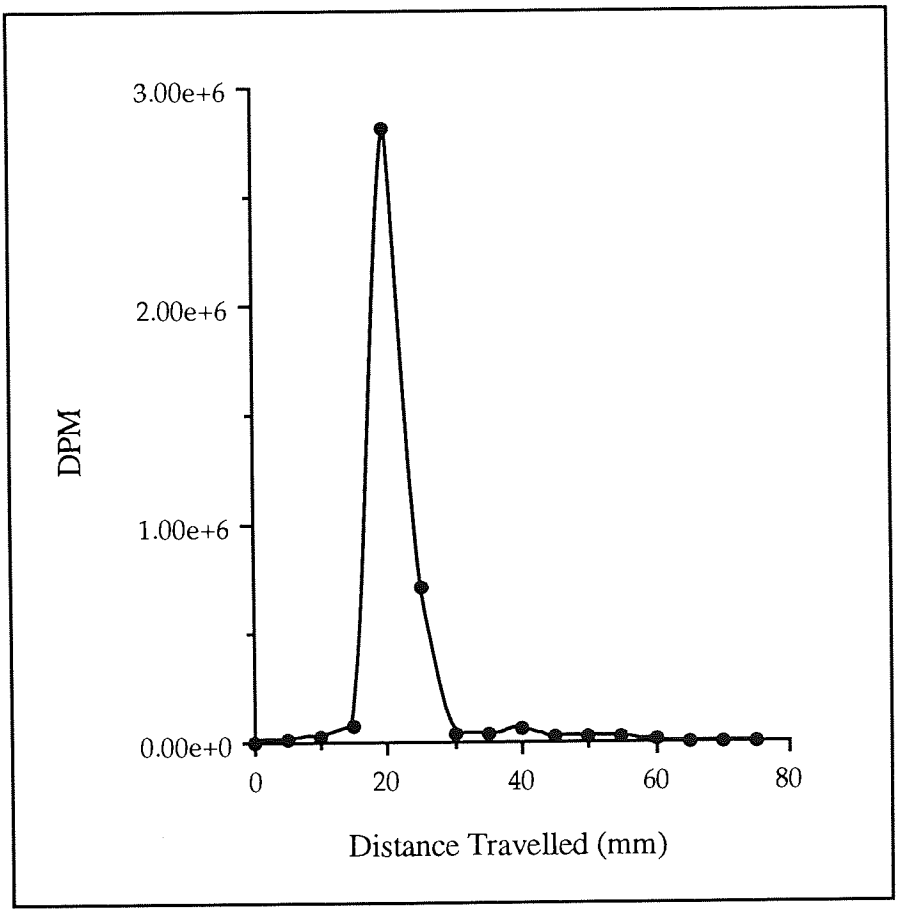
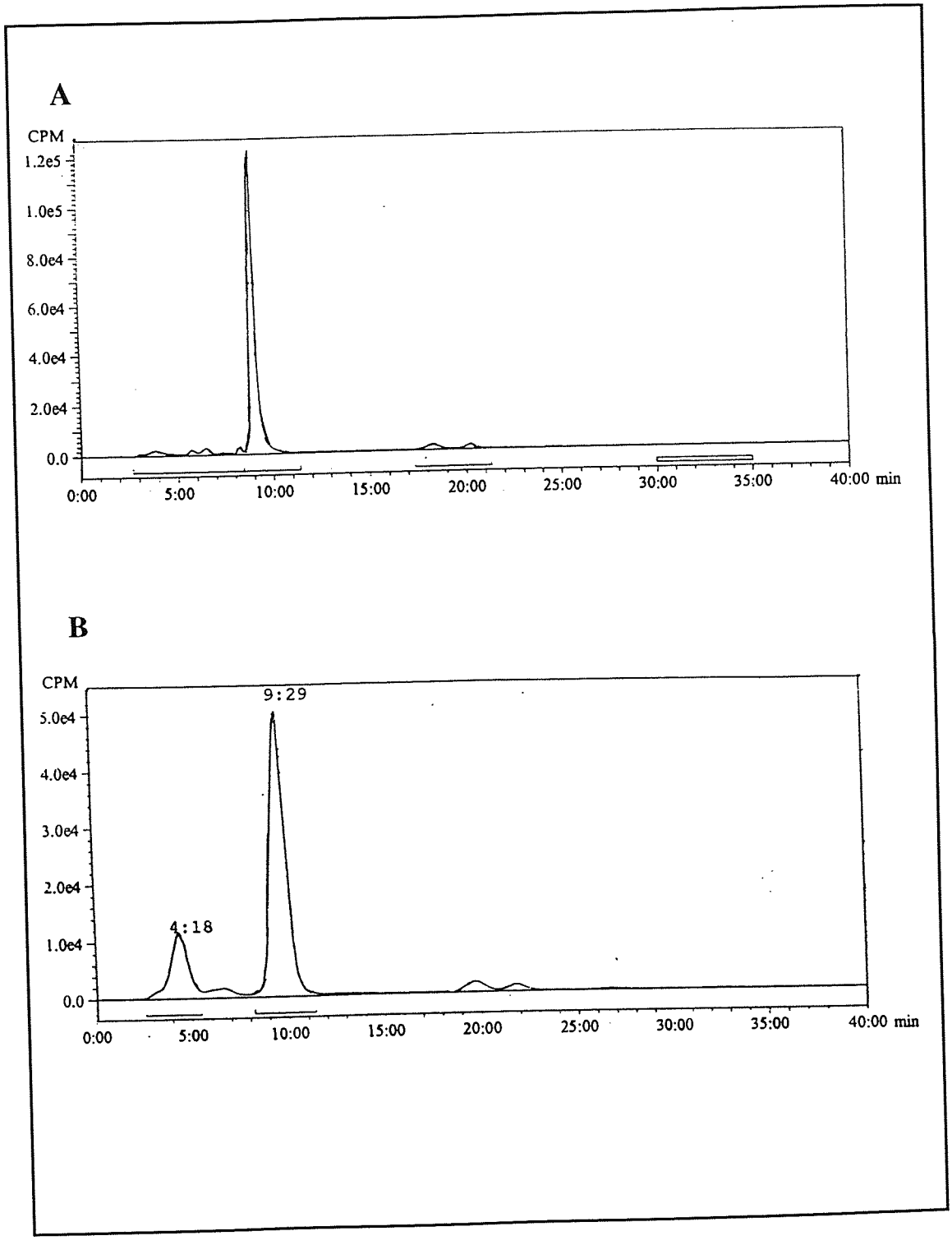


FIGURE 3.3 *Identification and purity assessment of Gly-[^{3}H]L-Pro*

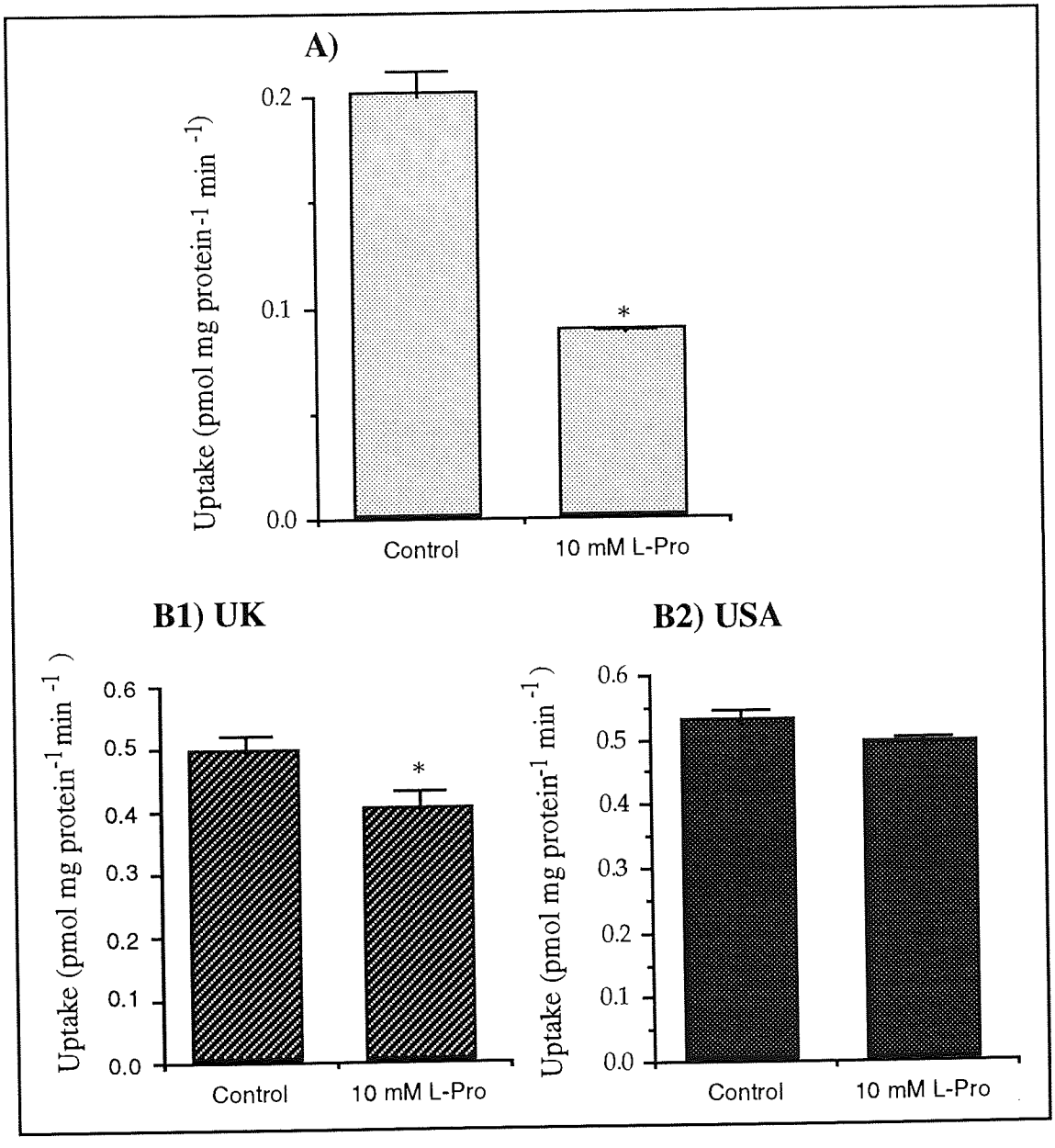
Radio-HPLC chromatogram showing the tritium-distribution of (**A**) Gly-[^{3}H]L-Pro, and (**B**) Gly-[^{3}H]L-Pro in relationship to [^{3}H]L-Pro.



Caco-2 cells have been reported to produce prolidase, the enzyme, which is responsible for X-Pro hydrolysis (X = amino acid) [Hu *et al.*, 1994a]. Prolidase is primarily an enzyme of cytosolic domain, which has been demonstrated to hydrolyse the dipeptide L-Phe-L-Pro. The majority of L-Phe-L-Pro was hydrolysed intracellularly, however, 2-5 % was hydrolysed in the apical compartment as a result of brush-border enzyme [Hu *et al.*, 1994a]. As enzyme activity in cells has been shown to change with respect to time [Beaulieu & Quaroni, 1991: Darmoul *et al.*, 1992], the possibility of enzymatic expression differing between cell clones, resulting in increased brush border expression of prolidase, had to be considered. Gly-L-Pro may be subject to hydrolysis at the apical membrane of the cells, resulting in the production of [^{3}H]L-Pro. Caco-2 cells have an active, saturable, imino-acid transport system [Nicklin *et al.*, 1992a] which is responsible for approximately 70 % of the uptake of [^{3}H]L-Pro into the cells at 50 mM. Therefore, the tritium in the cells may be due to both Gly-[^{3}H]L-Pro and [^{3}H]L-Pro, giving an over estimation of the dipeptide uptake. The imino-acid uptake displays concentration-dependency (K_m = 5.28 mM) and therefore unlabelled L-Pro, in excess of the K_m value (10 mM), was included in the incubation medium in order to block [^{3}H]L-Pro (that may arise from Gly-[^{3}H]L-Pro hydrolysis) uptake into the cells. Excess L-Pro inhibited [^{3}H]L-Pro uptake by 56.41 ± 1.09 % (Figure 3.4 A), indicating that the Caco-2 cell clone used within this study expresses an active imino-acid transport system. Our results indicate that approximately 55 % of uptake can be blocked, suggesting a passive component accounting for 45 % at 10 mM. Nicklin *et al.*, estimated about 70 % of uptake was due to an active process at 50 mM [Nicklin *et al.*, 1992a], suggesting that the transport system varies between cell clones. Gly-[^{3}H]L-Pro uptake in the presence of 10 mM L-Pro in the UK was significantly different from the control, producing 19.05 ± 6.74 % inhibition (Figure 3.4B1), whereas while in the USA, total uptake was only reduced by 6.68 ± 2.38% (not significant at 95 % confidence limits tested) (Figure 3.4B2). The effect of 10 mM L-Pro on uptake serves as an indicator to the extent of the hydrolysis (stability) of the probe compound, therefore, indicating that hydrolysis was negligible during the 3 min experimental period while at in the USA, but not while in the UK. The difference in % inhibition obtained for the two locations (UK versus USA), indicates that environmental factors, such as differences in growth media products and incubator conditions may effect the enzymatic expression of the cells. It also highlights the possibility that cell clones from different laboratories possess different characteristics. 10 mM L-Pro was included in the incubation solution for all future experiments in an to attempt to block any [^{3}H]L-Pro uptake into the cells and thus normalise conditions between cells clones and laboratories. Excess L-Pro has also been shown to inhibit prolidase activity by 66 % [Hu *et al.*, 1994a] and therefore acts as a preventative measure to hydrolysis. Excess L-Pro will also block the efflux of [^{3}H]L-Pro, liberated by cytosolic hydrolysis of Gly-[^{3}H]L-Pro, back into the apical phase, which would give inaccurate results.

FIGURE 3.4 *The effect of 10 mM L-Pro on the uptake of [^{3}H]L-Pro (A) and Gly-[^{3}H]L-Pro (B) into Caco-2 cell monolayers*

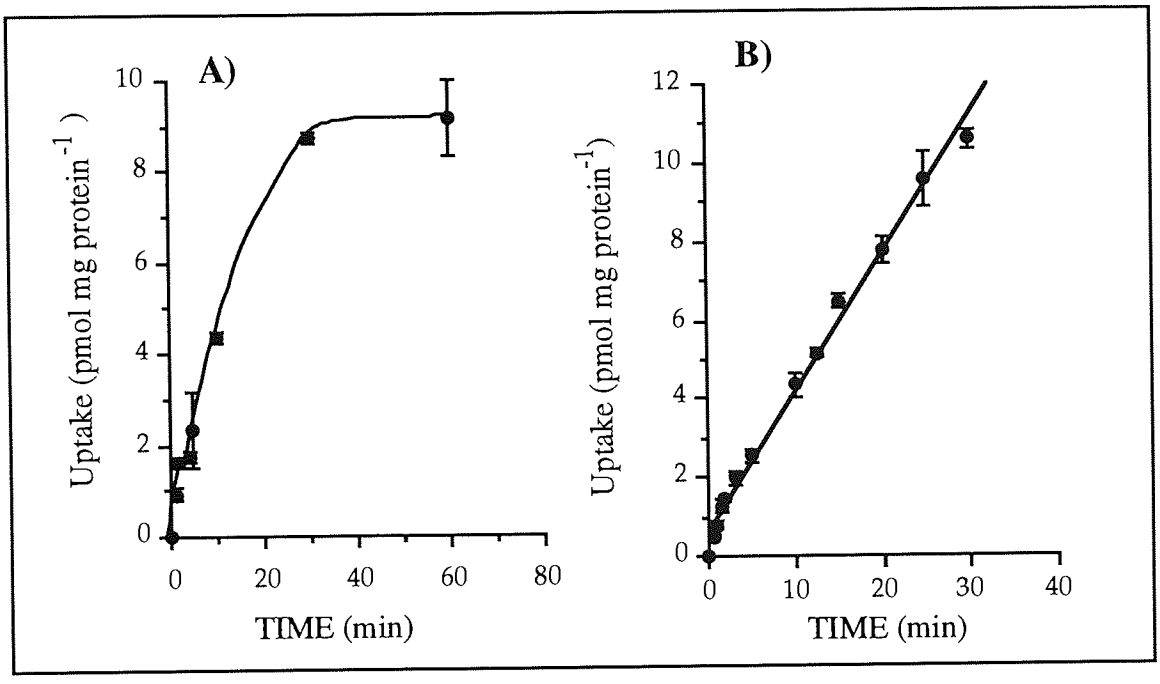
Monolayers were washed (1 x 500 µl x 5 min) at 37°C, with M4 at pH 6, before the incubation solutions were applied. **A**) 100 nM [^{3}H]L-Pro in the presence of 10 mM L-Pro, ,**B1**) 50 nM Gly-[^{3}H]L-Pro and 80 nM Gly-L-Pro in the presence of 10 mM L-Pro, while at the UK, or **B2**) 50 nM Gly-[^{3}H]L-Pro and 80 nM Gly-L-Pro in the presence of 10 mM L-Pro, while at the USA. Data are presented as mean values (of at least three monolayers) ± S.D. * Denotes a significant reduction ($p < 0.05$) from the control value.



The kinetic profile of Gly-[^{3}H]L-Pro uptake shows a relationship between uptake and time which deviates somewhat from linearity (Figure 3.5). Originally the study was carried out over a 60 min time period (A) on Caco-2 cell monolayers grown on 96-well plates. Uptake was found to plateau out after 30 min, and therefore the study was repeated using a shorter time frame. At 30 min, 5.32 % of Gly-[^{3}H]L-Pro in the donor phase had been taken up into the cells, indicating that a sink condition is not occurring and uptake is not reaching a plateau due to depletion of Gly-[^{3}H]L-Pro from the apical (donor) phase. An incubation period of 3 min was selected for the competition studies as it falls within the initial linear uptake phase and the relatively short incubation period minimises any hydrolysis of Gly-[^{3}H]L-Pro and the competitors that may occur. The use of 96-well plates was intended to produce a system that could incorporate a degree of automation. However, due to a reduction in the growing area the monolayers were more difficult to work with and prone to sloughing off the surface, and therefore future experiments were conducted using 24-well plates.

FIGURE 3.5 *Kinetics of Gly-[^{3}H]L-Pro uptake into Caco-2 cell monolayers*

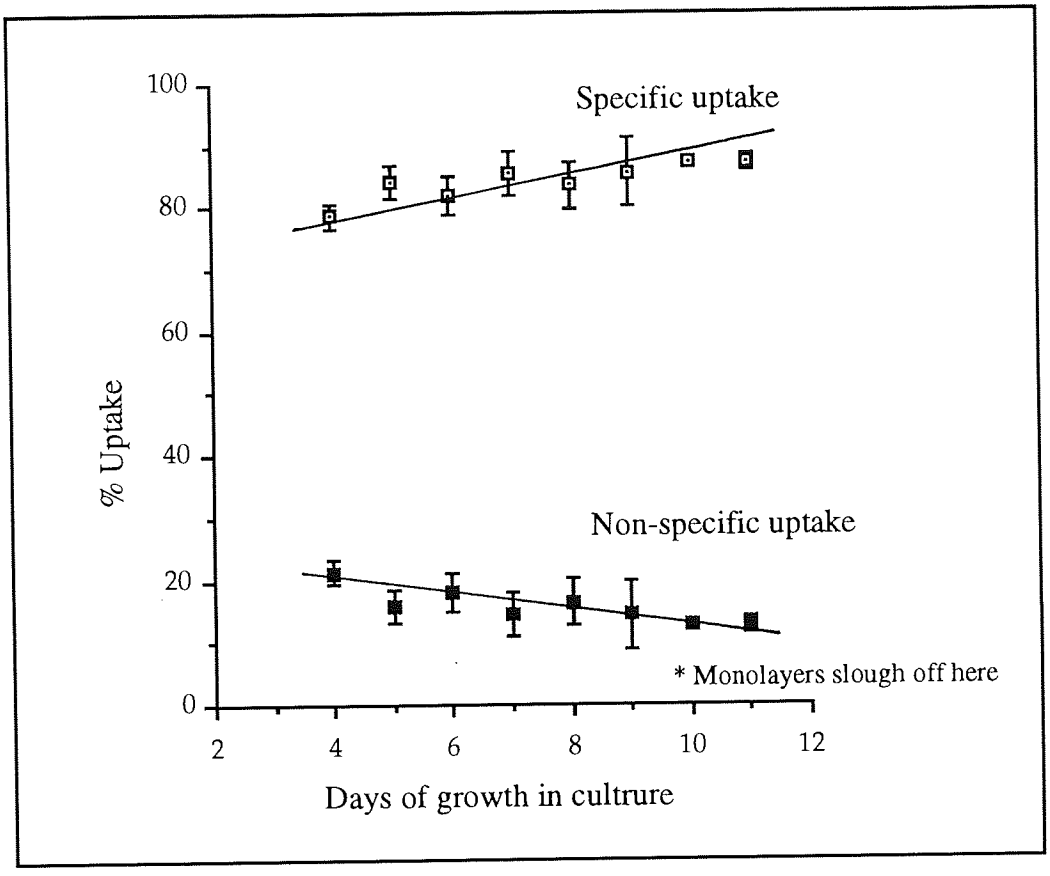
Monolayers were washed (1 x 200 µl x 5 min) for 96-well plates, and (1 x 500 µl x 5 min) for 24-well plates, at 37°C with M4 at pH 6. Incubation solution contained 10 mM L-Pro, 50 nM unlabelled Gly-L-Pro and 40 nM Gly-[^{3}H]L-Pro, in M4 at pH 6. Total uptake was determined after **A**) 1, 2, 4, 5, 10, 30 and 60 min, and **B**) 0.5, 1, 1.5, 2, 3, 5, 10, 12.5, 15, 20, 25 and 30 min, at 37°C. Data are mean values (from at least three monolayers) ± S.D.



It would be advantageous if the uptake of the dipeptide was studied when there was maximum expression of the DTS in the cells. The age of the monolayers (d after seeding) and the time taken for differentiation and development of transport systems after reaching confluency is an important factor. The specific (active) versus non-specific (passive) uptake of Gly-[^{3}H]L-Pro was studied over a period of 7 d (age 4-11 d) (Figure 3.6). At 4 d, the passive component was the greatest at 21.3 ± 2.06 % of total uptake at 5 mM substrate concentration. Throughout the 7 d period, the non-specific uptake decreased. Maximum uptake was seen at 11 d with the highest specific component (87 %) of the total uptake, however, after 10 d the monolayers became less adherent to the wells and began to slough off. The specific component was constant between the 7-9 d period accounting for > 85 % of total uptake, and adhesion of the cell monolayers to the wells were good. Total uptake increased from d 4-d 11, and, therefore 7-9 d was selected as the experimental window as the specific uptake is > 85 % and adhesion to the well is good. Although the difference in % specific uptake is not large between all experimental days, the total uptake increased significantly over the 7 d period 0.10 ± 0.003 pmol min^{-1} well^{-1} (d 4) to 0.30 ± 0.003 pmol min^{-1}well^{-1} (d 11) at 130 nM, and 0.78 ± 0.05 nmol min^{-1} well^{-1} (d 4) to 1.47 ± 0.16 nmol min^{-1} well^{-1} (d 11) at 5 mM). This suggests that the number of transporters expressed per monolayer is increasing over time, and, therefore the experimental window of 7-9 d post-seeding was selected. The diffusional (passive) component is not saturable and can not be blocked by excess Gly-L-Pro or competitors and, therefore, the diffusional component (15 %) was subtracted from total uptake when calculating IC_{50} values.

FIGURE 3.6 *Specific uptake of Gly-[^{3}H]L-Pro into Caco-2 cell monolayers*

Monolayers were washed (1 x 500 µl x 5 min) for 24-well plates, at 37°C with M4 at pH 6. Incubation solution contained 10 mM L-Pro, 80 nM Gly-[^{3}H]L-Pro and 50 nM or 5 mM unlabelled Gly-L-Pro, in M4 at pH 6. The specific uptake was calculated as a percentage of the total (uptake at 130 nM) by subtracting the % non-specific (uptake at 5 mM). Data are mean values (from at least three monolayers) ± S.D.



Previous studies using a variety of tissues and models (*e.g.* human, mouse, ovine , rat and rabbit intestinal BBMV, and Caco-2 cells) have revealed that Gly-L-Pro is transported by a carrier-mediated process [Rubino *et al.*, 1971: Lane *et al.*, 1975: Ganapathy *et al.*, 1981: Ganapathy & Leibach, 1983: Rajendran *et al.*, 1985a, 1985b: Wootton, 1986: Wootton & Hazelwood, 1989: Skopicki *et al.*, 1991: Backwell *et al.*, 1995]. Active uptake/transport systems display a saturable process (concentration-dependency). To examine the concentration-dependency of Gly-[^{3}H]L-Pro uptake, the amount of Gly-[^{3}H]L-Pro taken up into the cells at pH 6.0 was measured in the presence of unlabelled Gly-L-Pro over a concentration range of 0.001 to 10 mM. At 10 mM (10,000-fold molar excess of the unlabelled Gly-L-Pro) Gly-[^{3}H]L-Pro uptake was reduced considerably, but not eliminated (Figure 3.7). This indicated the presence of a saturable and non-saturable process, which was quantified when the data were fitted by non-linear regression using the Fig. P (Biosoft) computer program to the following equation:

EQUATION 3.1

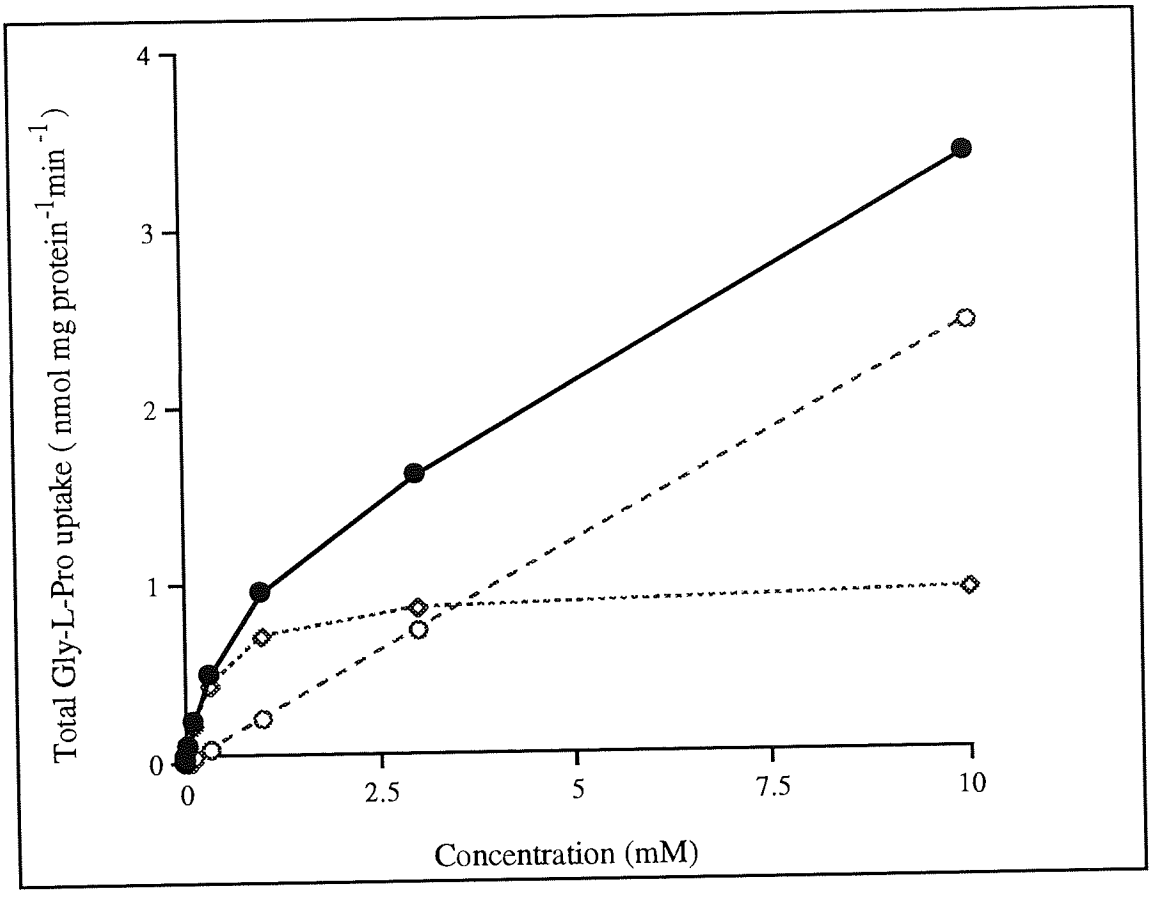
$$V = \frac{V_{max}[S]}{K_m + [S]} + k_d[S]$$

Where k_d = diffusional rate constant (nmol min^{-1} mg protein^{-1} mM^{-1}),
K_m = Michaelis-Menten constant (mM),
[S] = substrate concentration (mM),
V = flux (nmol min^{-1} mg protein^{-1}),
V_{max} = maximum rate of uptake (nmol min^{-1} mg protein^{-1}).

The kinetic parameters of the Gly-L-Pro uptake were: V_{max} of 0.983 ± 0.043 nmol min^{-1} mg protein $^{-1}$; K_m of 0.389 ± 0.015 mM; and k_d of 0.245 ± 0.005 nmol min^{-1} mg protein $^{-1}$ mM (r^2 = 0.9999). Thus, a saturable process which shows a high affinity for Gly-L-Pro is implicated in the uptake of Gly-L-Pro into Caco-2 cells, which is in agreement with previous literature. However, the kinetic parameters from the concentration-dependency study show that the characteristics of the system differ from values previously reported for other models. Gly-L-Pro uptake in Caco-2 cells displays a higher affinity (K_m = 0.389 ± 0.015 mM) for the transporter than Gly-L-Pro uptake in human, (K_m = 4.1 ± 0.5 mM) [Rajendran *et al.*, 1985a], mouse (K_m = 30.8 ± 1.9 mM) [Rajendran *et al.*, 1985b], and rabbit (K_m = 3.5 ± 0.36 mM) [Wootton, 1986] BBMV. However, Gly-L-Pro transport in an ovine model (K_m = 4.84 ± 0.82 μM), displays the highest affinity for the transporter [Backwell *et al.*, 1995]. The passive component seen in rabbit BBMV was 19 % at 100 mM Gly-L-Pro [Wootton, 1986], which falls within the range reported here (15.1 ± 3.91 % at 5 mM). There are no kinetic values for Gly-L-Pro uptake into Caco-2 cells available in the literature. Therefore, it is difficult for a direct comparison to be made, as previous studies have examined Gly-L-Pro uptake using different experimental models, tissues and conditions.

FIGURE 3.7 *Concentration-dependency of Gly-[^{3}H]L-Pro uptake into Caco-2 cells monolayers*

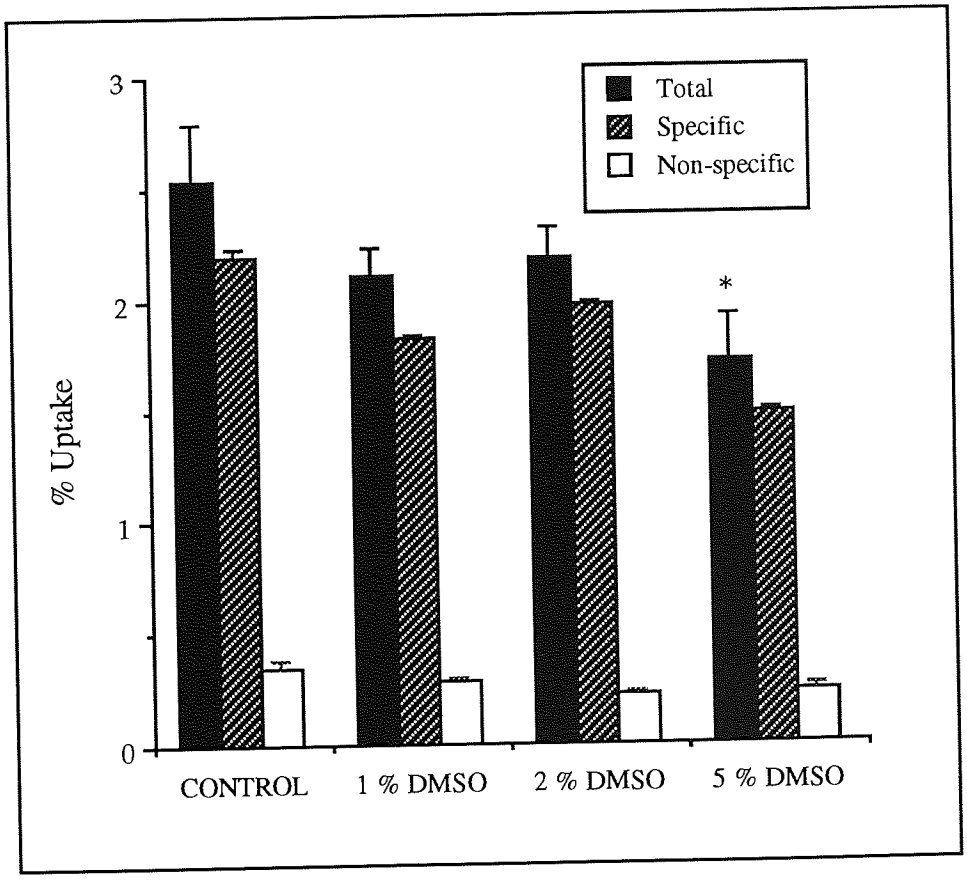
The monolayers were washed (1 x 500 µl x 5 min), with M4 at pH 6, and then incubated with M4 containing 50 nM Gly-[^{3}H]L-Pro and 10 mM L-Pro in the presence of increasing concentrations of unlabelled Gly-L-Pro (0.001 mM to 10 mM). The data points for the total uptake (•) are experimental values and expressed as nmol mg protein^{-1} min^{-1} (mean ± S.D. of at least three monolayers). The active (◇) and passive (O) component of total uptake are theoretical plots calculated using the Fig. P (Biosoft) computer program, which produced kinetics values of V_{max} = 0.983 ± 0.043 nmol min^{-1} mg protein^{-1}; K_m = 0.389 ± 0.015 mM; and k_d = 0.245 ± 0.005 nmol min^{-1} mg protein^{-1} mM (r^2 = 0.999). Error bars are too small to be displayed.



DMSO was selected as a cosolvent to in order to overcome solubility problems that could be encountered in future experiments when using poorly soluble competitors. The presence of the cosolvent DMSO at 1, 2 and 5 % did not significantly reduce the specific uptake (Figure 3.8); However, at 5 % DMSO concentration, there was approximately 30 % reduction in the total uptake. Therefore, a 2 % concentration of DMSO was selected, and included as the cosolvent in all incubation solutions, as it did not appear to have any effect on the uptake of the dipeptide, and did not affect the cells.

FIGURE 3.8 *The effect of DMSO on uptake of Gly-[^{3}H]L-Pro into Caco-2 cell monolayers*

The monolayers were washed (1 x 500 µl x 5 min) at 37°C, with M4 at pH 6, and then incubated with M4 containing 10 mM L-Pro and 50 nM Gly-[^{3}H]L-Pro, in the presence of 0 (control) 1, 2 or 5 % DMSO. The experiment was then repeated in the presence of 5 mM unlabelled Gly-L-Pro. The % specific uptake was calculated by subtracting the % non-specific (uptake at 5 mM) from the total (uptake at 50 nM). Data are mean values (from at least three monolayers) ± S.D. * Denotes a significant ($p < 0.05$) reduction from the control value.



Gly-[^{3}H]L-Pro uptake was significantly reduced by decreasing the temperature below 37°C (29.61 ± 1.63, 43.10 ± 5.37, 63.37 ± 2.15 and 93.76 ± 0.32 % reductions at 30.5, 25.5, 19 and 4°C, respectively; Figure 3.9 A). An E_a of 46.67 kJ.mol^{-1} was calculated for the active component of Gly-[^{3}H]L-Pro uptake, by linear-squares regression analysis of a plot of ln k against 1/T according to the Arrhenius equation (Figure 3.9 B and Equation 3.2 below) for these data.

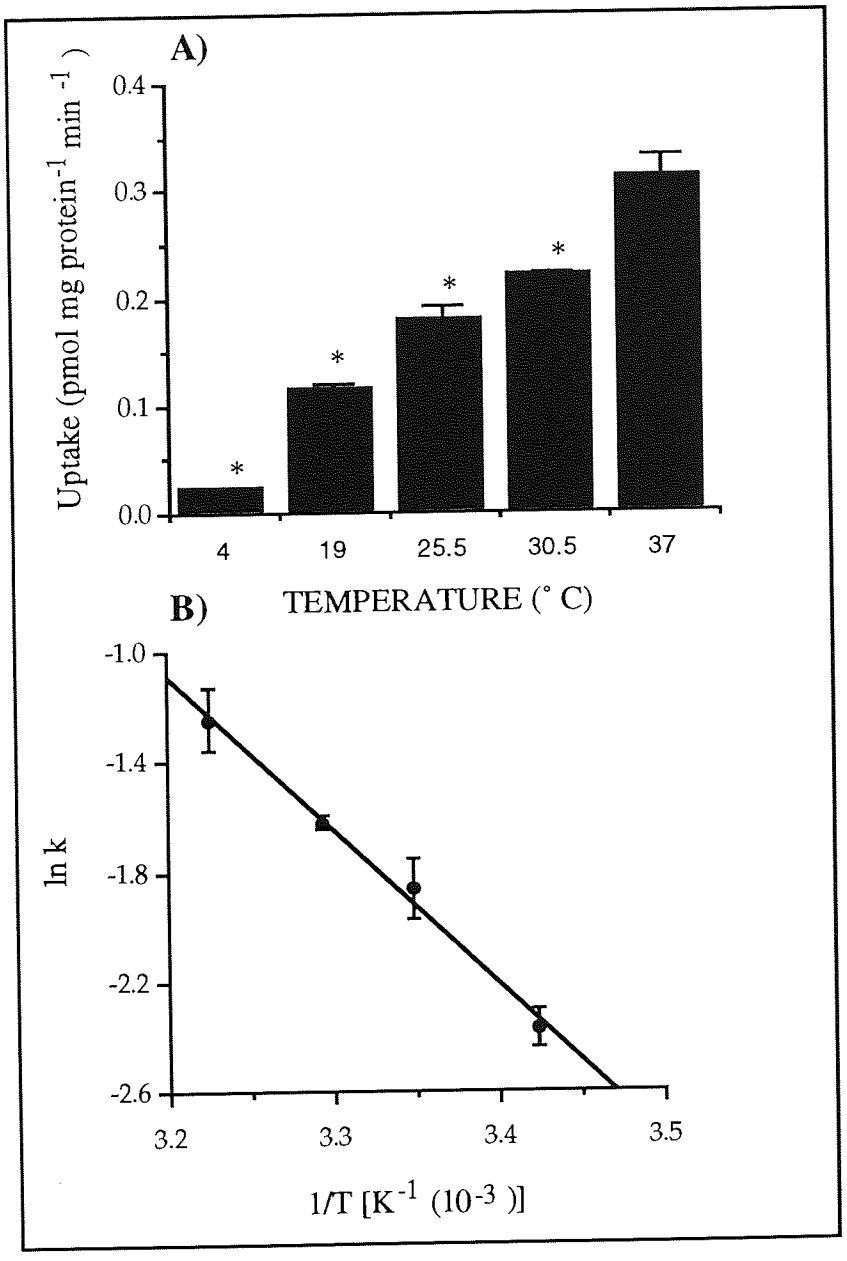
EQUATION 3.2

$$\ln k = \ln A - \frac{E_a}{RT}$$

The calculated E_a exceeds that anticipated for simple diffusion (< 16.8 kJ.mol^{-1}) and falls within the range reported for an active carrier-mediated process (29.4 - 105.0 kJ.mol^{-1}) [Hopfer & Hogget, 1981]. This provides further evidence for the involvement of an active transport process in Gly-[^{3}H]L-Pro uptake into Caco-2 cell monolayers.

FIGURE 3.9 *Temperature-dependency of Gly-[^{3}H]L-Pro uptake into Caco-2 cell monolayers*

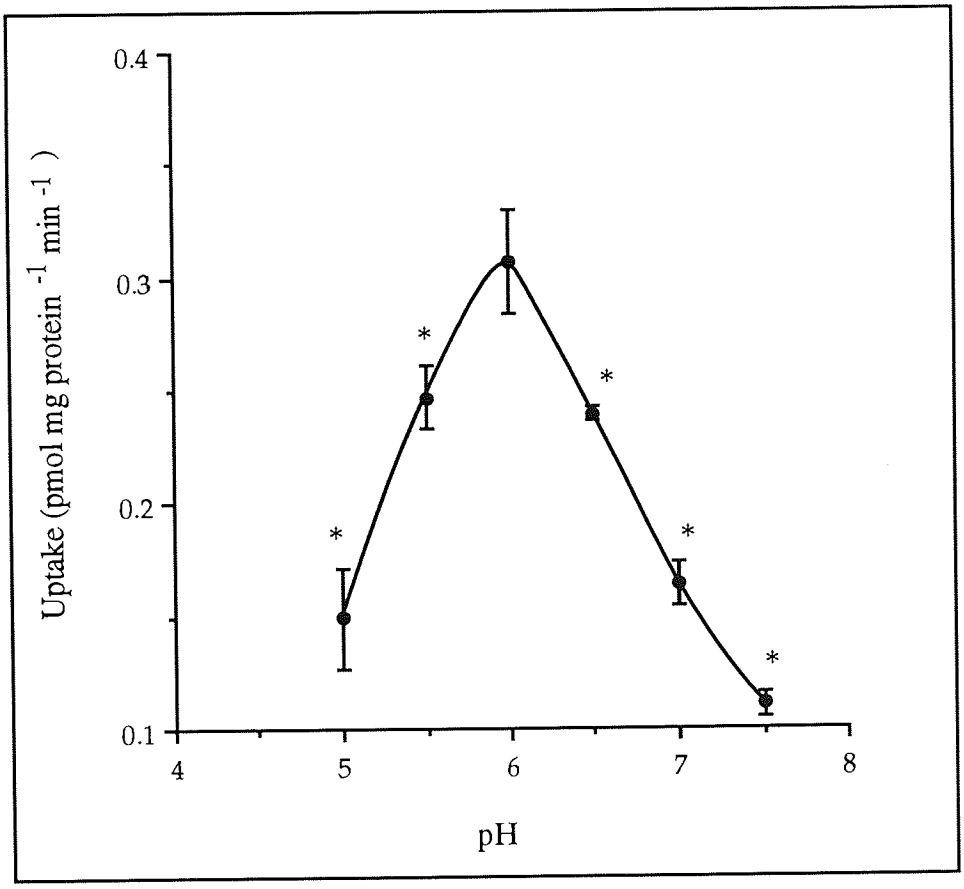
(A) Monolayers were washed (1 x 500 µl x 30 min) with M4 pre-equilibrated at 4, 19, 25.5, 30.5, or 37°C, as appropriate. Uptake of Gly-[^{3}H]L-Pro was determined at 4, 19, 25.5, 30.5, and 37°C. Data are expressed as mean (of at least three monolayers) ± S.D. * Denotes a significant ($p < 0.05$) reduction form control (uptake at 37°C). **(B)** The activation-energy for Gly-[^{3}H]L-Pro uptake was calculated from an Arrhenius plot of these data, giving a value of 46.67 kJ.mol^{-1} ($r^2 = 0.993$).



The optimum pH for dipeptide proton-coupled transport into the cells has been reported to be pH 6 [Ganapathy & Leibach, 1983: Thwaites *et al.*, 1993a, b, c, g, 1994a], a value which is within the range for the small intestine [Kararli, 1989]. Gly-[^{3}H]L-Pro uptake in our system displayed a definite pH-dependence (Figure 3.10), producing a "classical" bell shaped curve. The optimum pH for maximum uptake was pH 6, which is in agreement with previous reports. Conditions which were more acidic or alkali produced a significant reduction in uptake. The pH-dependence can be explained due to the DTS being driven by a proton gradient, with optimum pH for the driving force being pH 6 [Thwaites *et al.*, 1993a, b, c, g, 1994a].

FIGURE 3.10 *pH-dependency of Gly-[^{3}H]L-Pro uptake into Caco-2 cells monolayers*

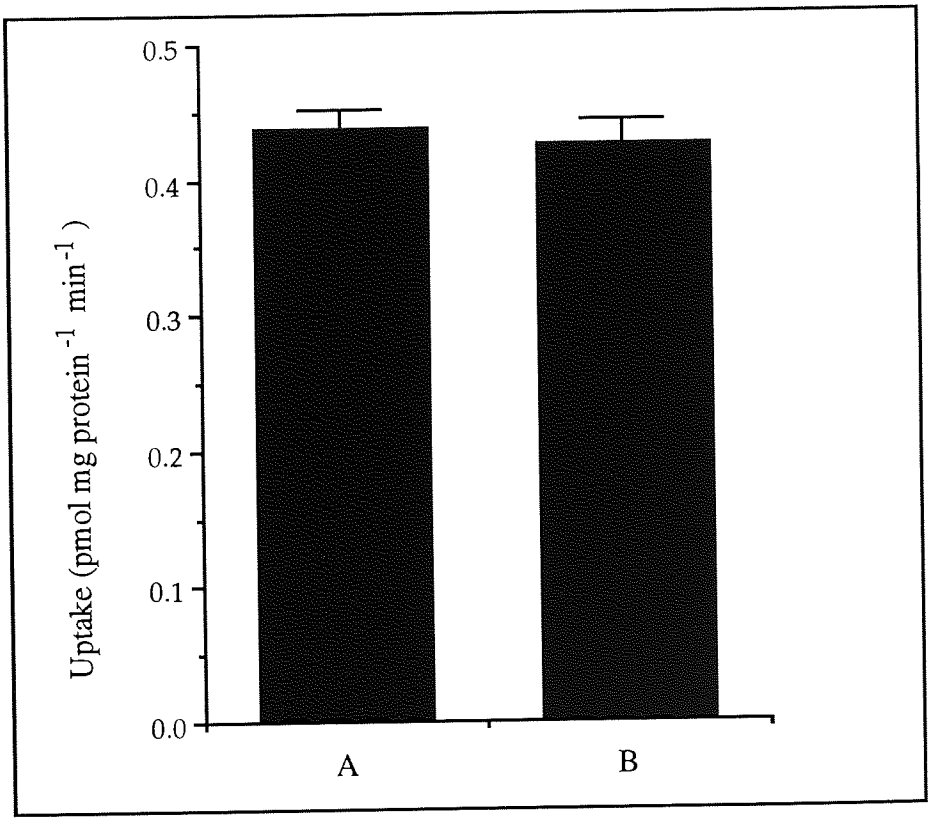
Monolayers were washed (1 x 500 µl x 30 min) with M4 or M5 buffered at the appropriate pH (5.0, 5.5, 6.0, 6.5, 7.0, and 7.5), at 37 °C. Uptake of Gly-[^{3}H]L-Pro over a 3 min period, was determined at a range of pH values (pH 5.0 to pH 7.5). Data are expressed as mean (of at least three monolayers) ± S.D. * Denotes a significant ($p < 0.05$) reduction from Gly-[^{3}H]L-Pro uptake at pH 6.0.



The standard protocol for uptake studies requires the monolayers to be washed prior to incubation, in order to remove any dead/floating cells, serum components or ions that are not required within the incubation media, and to achieve the desired conditions for experimentation (temperature/pH of cells). Washing protocols are usually 15 - 30 min depending on the individual experiment. The effect of decreasing the pre-incubation wash from 15 to 5 min (Figure 3.11) had no significant effect on the total uptake of Gly-[^{3}H]L-Pro. Therefore, a 5 min pre-incubation washing period was selected for future experiments.

FIGURE 3.11 *The effect of the pre-incubation washing protocol on Gly-[^{3}H]L-Pro uptake into Caco-2 cell monolayers*

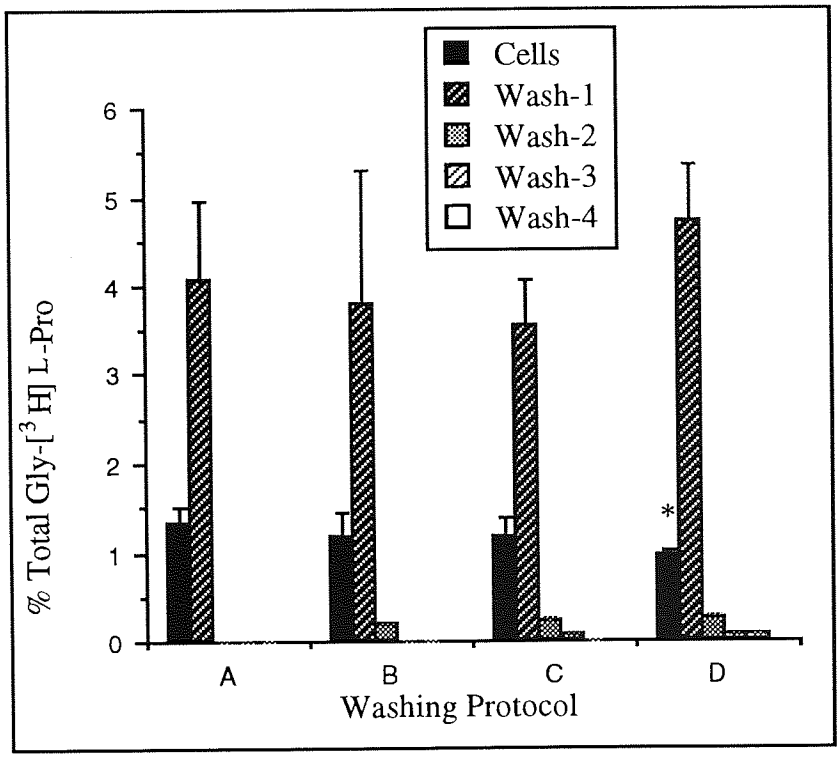
Monolayers were washed using the following protocols; **A**) 1 x 500 µl x 5 min, or **B**) 1 x 500 µl x 15 min, with M4 at pH 6, at 37°C. Uptake of 50 nM Gly-[^{3}H]L-Pro in the presence of 10 mM L-Pro, in M4 containing 2 % DMSO, was then compared for **A** and **B**. Data are expressed as mean (of at least three monolayers) ± S.D.



In order to assess that Gly-[^{3}H]L-Pro uptake into the cells is a true value, it is necessary to ensure that Gly-[^{3}H]L-Pro bound non-specifically to the cell membrane is removed during the post-incubation washing procedure. The effect of the post-incubation washing protocol (number of washes, 1 - 4 x 5 min) was investigated (Figure 3.12). Protocol D (4 x 500 µl x 5 min) produced a significant reduction in total uptake from the control (A). Increasing the number of washes resulted in the monolayers becoming susceptible to damage and sloughing was apparent, leading to a reduction in the total uptake, as seen in D. Protocol B (2 x 500 µl x 5 min) was selected as this procedure removed 97.80 % of the non-specifically bound label from the cell surface, and no damage to monolayers occurred.

FIGURE 3.12 *The effect of the post-incubation washing protocol on Gly-[^{3}H]L-Pro uptake into Caco-2 cell monolayers*

Monolayers were washed (1 x 500 µl x 5 min) at 37°C, with M4 at pH 6 and incubated (1 x 250 µl x 3 min) with 10 mM L-Pro and 50 nM Gly-[^{3}H]L-Pro in M4 (2 % DMSO) at pH 6. The monolayers were then washed with PBS-azide at 4°C, using the following protocols; A) 1 x 500 µl x 5 min, B) 2 x 500 µl x 5 min, C) 3 x 500 µl x 5 min and D) 4 x 500 µl x 5 min. Total uptake for the protocols and ^{3}H content of each wash was then compared. Data are expressed as mean (of at least three monolayers) ± S.D. * Denotes a significant ($p < 0.05$) reduction in total uptake from the control value (protocol A).

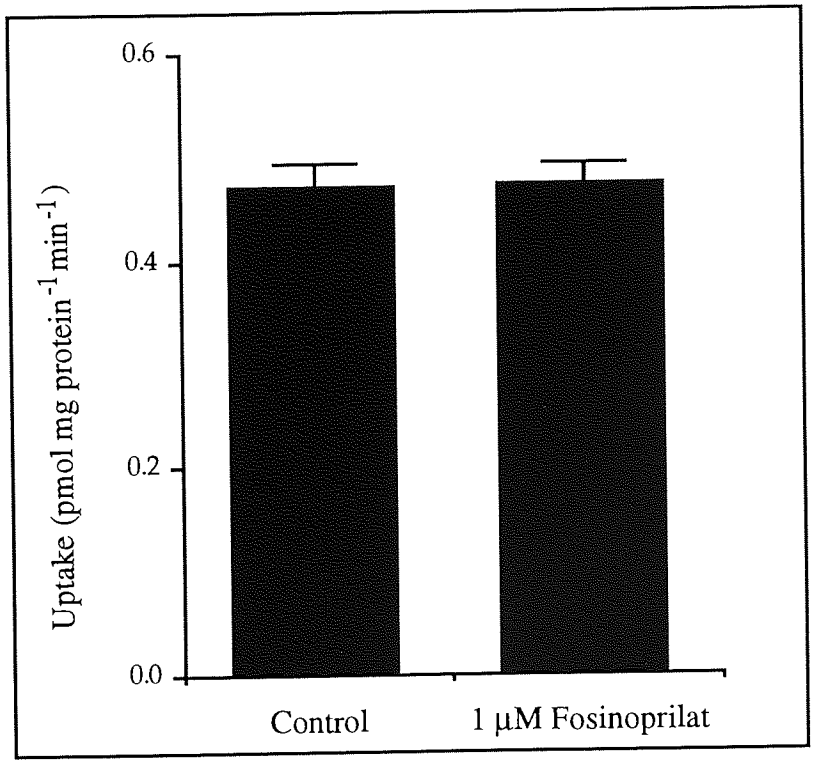


3.3.2 SPECIFICITY OF GLY-[^{3}H]L-PRO UPTAKE INTO CACO-2 CELL MONOLAYERS

Gly-L-Pro is a weak ACE-inhibitor (IC_{50} = 450 μM at pH 8.3) [Cheung *et al.*, 1980]. Therefore, it is a possible that Gly-L-Pro may bind to ACE, which is expressed in a heterogeneous mosaic pattern on the apical membrane of Caco-2 cells [Howell *et al.*, 1993]. Fosinoprilat is an ACE-inhibitor with a high affinity for the enzyme (IC_{50} = 180 nM) [Krapcho *et al.*, 1988]. It has been reported to be absorbed *via* a passive process in rats, and therefore, should have no interaction with the DTS [Friedman & Amidon, 1989b]. If Gly-[^{3}H]L-Pro was binding to ACE, the presence of fosinoprilat at a concentration of 1 μM, which is greater than 5 times its IC_{50} value for ACE, would cause inhibition of Gly-[^{3}H]L-Pro uptake. Uptake in the control was 0.559 ± 0.076 pmol mg protein^{-1} min^{-1}, compared to 0.607 ± 0.030 pmol mg protein^{-1} min^{-1} in the presence of 1 μM fosinoprilat (Figure 3.13). An unpaired t-test (t = 1.03) shows that the values are not significantly different. Therefore, this suggests that, at the concentration used in the experiments, the Gly-[^{3}H]L-Pro is not binding to ACE.

FIGURE 3.13 *The effect of 1 μM fosinoprilat on Gly-[^{3}H]L-Pro uptake into Caco-2 cell monolayers*

The monolayers were washed (1 x 500 μl x 5 min) with M4 at pH 6, at 37°C. The effect of fosinoprilat on uptake of 50 nM Gly-[^{3}H]L-Pro was investigated, by coadministering 1 μM with the control. Data are expressed as mean (of at least three monolayers) ± S.D.



In order to check that the system is reproducible among experimental days, and to assess the effect of cell age (with respect to passage number) on the DTS, the ACE-inhibitor, SQ-29852, which has been shown to utilise the DTS in its uptake into Caco-2 cells [Marino *et al.*, 1996: Nicklin *et al.*, 1996], was used as a competitor. The mean ± S.D. % inhibition and IC_{50} ± S.D. values for 16 different experiments (mean value) which use cells of increasing passage number (25 - 40) were 60.51 ± 6.73 % and 0.68 ± 0.17 mM respectively, thus showing the system is reproducible (Table 3.1).

TABLE 3.1 *Reproducibility of the system; SQ-29852 inhibition of Gly-[^{3}H]L-Pro uptake into Caco-2 cell monolayers.*

Passage number	Inhibition % Mean [S.D.]	IC_{50} mM Mean [S.D.]
25	55.50 [1.73]	0.80 [0.06]
26	63.69 [7.20]	0.58 [0.18]
27	52.57 [3.14]	0.91 [0.11]
28	55.24 [2.87]	0.81 [0.10]
29	61.08 [0.47]	0.64 [0.13]
30	55.99 [1.65]	0.79 [0.05]
31	53.39 [0.07]	0.87 [0.02]
32	61.91 [0.78]	0.62 [0.02]
33	61.89 [8.83]	0.64 [0.22]
34	56.66 [1.33	0.77 [0.04]
35	56.18 [2.32]	0.78 [0.03]
36	57.29 [1.57]	0.75 [0.05]
37	69.36 [0.59]	0.44 [0.01]
38	59.73 [2.10]	0.74 [0.03]
39	74.25 [1.86]	0.35 [0.03]
40	73.40 [4.29]	0.37 [0.08]

1 mM SQ-29852 was included as a competitor of Gly-[^{3}H]L-Pro uptake on a number of experimental d in order to assess the reproducibility of the system. The effect of passage number on the % inhibition of 1 mM SQ-29852 was also investigated. Data are expressed as mean (of at least three monolayers) ± S.D.

IC_{50}, values, which is the concentration of the inhibitor that produces 50 % inhibition of uptake, were calculated by correcting the % inhibition for the passive component of uptake by subtracting the amount of uptake seen in the presence 5 mM unlabelled Gly-L-Pro (15 %) (see appendix 2). The transport inhibition constant (K_i mM) is related to the observed inhibition of carrier-mediated uptake using the ratio (r) of the uptake rate in the absence (V_a) of a competitor, to the value in the presence of (V_i) a competitor ($r = V_a/V_i$), according to Equation 3.3 [Nicklin *et al.*, 1995]:

EQUATION 3.3

$$K_i = \frac{K_m [I]}{(r - 1)\ (K_m + [S])}$$

when $K_m >> [S]$

$$K_i = \frac{K_m [I]}{(r - 1)\ K_m} = \frac{[I]}{(r - 1)}$$

or, when $r = 2$, $[I] = IC_{50}$,

$$IC_{50} = K_i$$

where K_m is Michaelis constant (mM) and [S] and [I] are the concentrations of substrate and competitor (mM), assuming that the compounds inhibit uptake competitively. In the present study of the inhibition of Gly-[^{3}H]L-Pro uptake, $K_m >> [S]$ and, at 50 % inhibition ($r = 2$, $[I] = IC_{50}$), this provides $K_i = IC_{50}$. The IC_{50} of competitors was thus estimated from:

EQUATION 3.4

$$IC_{50} = \frac{[I]}{(r - 1)}$$

Recently, a strong correlation between K_i and IC_{50} values has been experimentally determined when investigating competitive inhibition [Eddy *et al.*, 1995], thus confirming the above equations.

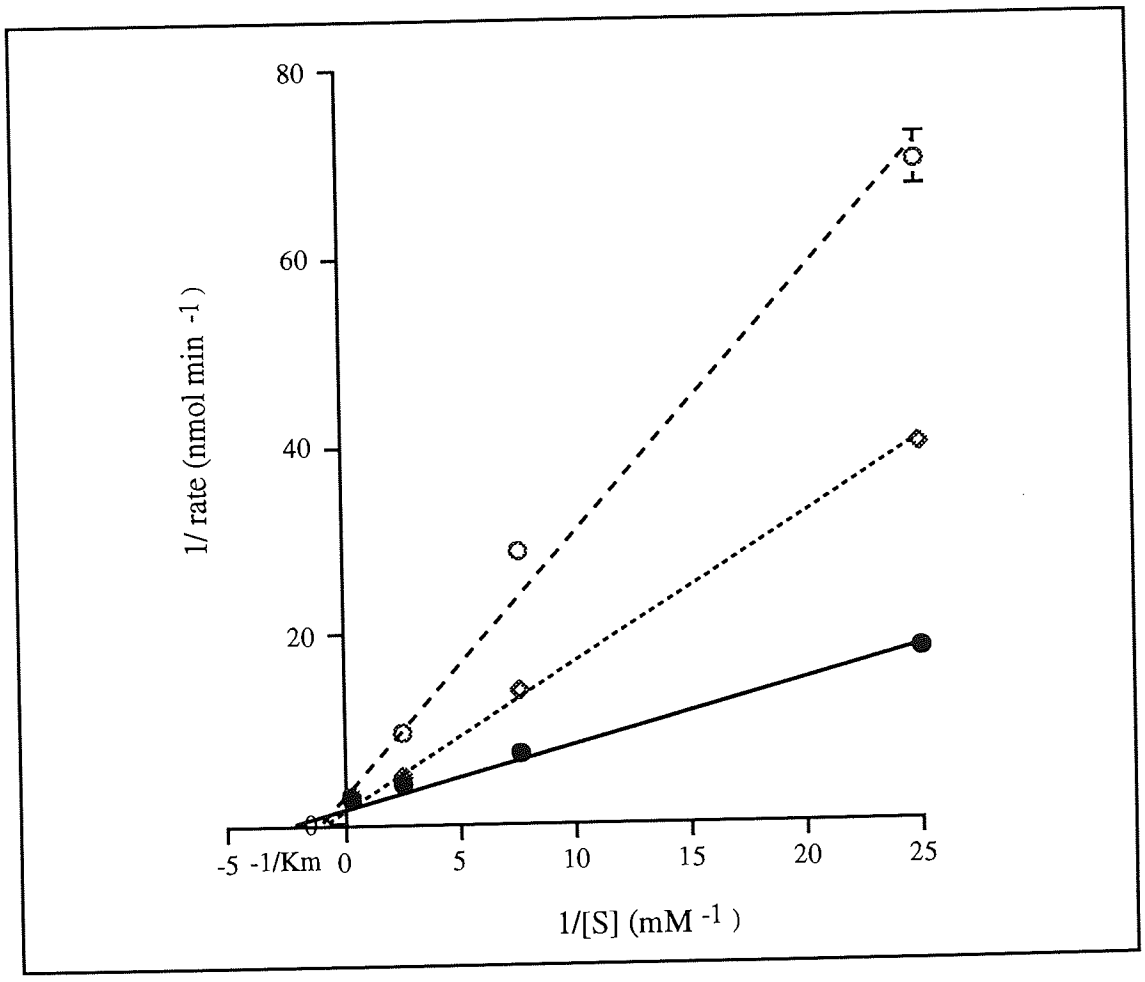
After passage number 38, there was a noticeable increase in inhibition, suggesting that the DTS is affected by cell age and the active component is increased. However, the growth of cells at passage 39 and above, is slower than younger cells and also trypsinisation becomes difficult. It has also been reported that after passage 35 mannitol transport across Caco-2 cells is increased suggesting a change is cell morphology [oral communication, Chong]. Therefore future, studies will use cells from passage 25 - 38 only.

SQ-29852 caused a decrease in the total Gly-[^{3}H]L-Pro taken up into the cells (Table 3.1). Therefore, in order to distinguish whether the SQ-29852 is displacing the Gly-[^{3}H]L-Pro in a competitive or non-competitive mechanism, a range of Gly-L-Pro concentrations

(0.13, 0.4, 3.6 and 49.6 mM) were examined in the presence of 0, (control) 1 and 3 mM SQ-29852. The data were corrected for non-specific uptake (passive) and the results were processed as a Lineweaver-Burk plot (Figure 3.14). All three lines crossed the y-axis at the same proximity (control, y = 1.415; 1 mM SQ-29852, y = 1.093; 3 mM SQ-29852, y = 2.416) indicating a competitive uptake mechanism. This also validates the use of IC_{50} values as competitive inhibition is occuring. The equation of the control plot was, y = 0.681x + 1.42, giving a K_m of 0.481 mM, which is in the same range as that reported for the concentration-dependency study.

FIGURE 3.14 *The binding mechanism of SQ-29852 to the DTS in Caco-2 cells monolayers*

Lineweaver Burk plot of SQ-29852 inhibition of Gly-[^{3}H]L-Pro uptake into Caco-2 cells. The monolayers were washed (1 x 500 µl x 5 min) with M4 at pH 6, then incubated with M4 containing 50 nM Gly-[^{3}H]L-Pro and 10 mM L-Pro in the presence of 0 (O), 1 (◇) or 3 (•) mM SQ-29852. The results (mean of at least three monolayers) are corrected for non-specific uptake and plotted according to $1/V_0$ (1/rate) = K_m/V_{max} (1/S) + $1/V_{max}$, where [S] = Gly-L-Pro concentration and the rate units are nmol min^{-1}.



The substrate specificity of the DTS was explored by comparing the uptake of Gly-[^{3}H]L-Pro (50 nM) alone or in the presence of 1 mM concentrations of various compounds that utilise a variety of transport mechanism in intestinal enterocytes. The inhibition of Gly-[^{3}H]L-Pro uptake in the presence of these compounds is reported in Table 3.2. The results are expressed as % ± S.D. inhibition of the control after correction for 15 % non-specific uptake (passive component). The anti cancer agent, bestatin, which is a synthetic dipeptide-like protease inhibitor, has been reported to be taken up into the cell *via* the DTS in rabbit intestinal brush-border membranes and Gly-L-Pro at a concentration of 11 mM has been shown to inhibit bestatin uptake by 59 % [Inui *et al.*, 1991]. About half of bestatin uptake was shown to be binding to the brush-border membranes, and therefore, Gly-L-Pro at the concentration tested is saturating the system, producing maximum inhibition [Inui *et al.*, 1991]. Tomita *et al.* using rabbit intestinal BBMV, have also shown that bestatin is a substrate for the DTS as it inhibits cephradine uptake in a competitive manner, and produced a countertransport effect on uptake [Tomita *et al.*, 1990]. When 1 mM bestatin was co-administered with Gly-[^{3}H]L-Pro in the present system, 67.4 ± 1.69 % inhibition was seen, which is within the range of inhibition produced by 11 mM Gly-L-Pro in the previous study [Inui *et al.*, 1991]. Results indicate that bestatin shares a common uptake system with Gly-L-Pro, thus providing evidence that the DTS is the system utilised.

The hydrophobic linear tripeptide glutathione (γ-glutamyl-cystyl-glycine {γ-Glu-Cys-Gly}), forms conjugates with chlorodinitronenzene which enters the cell by diffusion. The conjugates are then secreted out of the cell by an active-transport system which is present in both the apical and basolateral plasma membranes [Oude Elferink *et al.*, 1985]. Glutathione produced significant inhibition of the probe. The literature on this subject is contradictory. Kramer *et al.* have shown the existence of a membrane glycoprotein of 127 kDa, which is directly involved in the uptake of small peptides and orally active cephalosporins [Kramer *et al.*, 1990b]. A protein of similar weight, 120 ± 10 kDa, has been isolated from the membrane of Caco-2 cells [Dantzig *et al.*, 1994b]. Kramer *et al.* have demonstrated strong inhibition of the photoaffinity labelled 127 kDa protein by glutathione [Kramer *et al.*, 1995], and it has also been shown that di/tripeptides inhibited transport of glutathione across rat intestinal everted sacs [Hunjan & Evered, 1991], suggesting that it shares a common peptide intestinal transport system. However, no inhibition of Gly-L-Pro uptake by glutathione was seen in mouse intestinal BBMV model [Rajendran *et al.*, 1985b], which is supported by the finding that the γ-linkage in glutathione leads to poor transport [Matthews, 1987]. 25.4 ± 2.84 % inhibition of Gly-[^{3}H]L-Pro uptake was seen in the present study which is in agreement with the majority of literature, suggesting that glutathione uptake to some extent is *via* the DTS.

Literature on the transport of tetrapeptides by the DTS is somewhat controversial. Originally, the DTS has only been reported to transport small peptide units such as di- and tripeptides [Addison *et al.*, 1975a, b]. The tetrapeptide Gly-L-Pro-Gly-Gly produced considerable inhibition of the probe (41.7 ± 0.767 %). Three mechanisms may possibly be

responsible for the observed result. Tetrapeptides are the preferred substrates for the brush-border proteases [Kania *et al.*, 1972] and it may be that Gly-L-Pro-Gly-Gly is being hydrolysed into subunits (di- and tripeptides), and it is these that are responsible for the inhibition. However, L-Pro and Gly containing peptides are hydrolysis-resistant and, therefore, suggesting that the tetrapeptide is stable, and thus a substrate for the DTS. Tri/tetra/pentaglycine have been shown to inhibit the photoaffinity labelling of the 127 kDa membrane glycoprotein and also cephalexin uptake into rabbit BBMV [Kramer *et al.*, 1995]. The inhibitory effect was related to the length of the peptide chain, with the more amino acid residues present, the lesser the effect. This evidence suggests that the DTS is capable of transporting tetrapeptides. However, tetraglycine showed no inhibition of Gly-Sar intracellular accumulation and transepithelial transport in Caco-2 cell monolayers [Thwaites *et al.*, 1994a]. Therefore, the tetrapeptide may simply be blocking the binding site on the DTS, and thus preventing the probe from being taken up.

The metabolic inhibitor sodium azide blocked all of the active Gly-[^{3}H]L-Pro uptake after correction for the non-specific component (passive); this indicates an energy-dependent process. The energy is probably required to fuel the Na^+/H^+-antiporter, which maintains the proton gradient across the membrane in intestinal cells [Hoinard *et al.*, 1988]. The absorption mechanisms of TRH are somewhat controversial (section 1.8.4). Our results indicate that TRH is not a substrate for the DTS and are in agreement with Walter & Kissel, who found TRH to be transported exclusively *via* a paracellular passive route in Caco-2 cells of passage number 30-34 [Walter & Kissel, 1994]. The bile acid, taurocholic acid, which is actively transported *via* a Na^+-dependent system in Caco-2 cells [Hidalgo & Borchardt, 1990b], did not significantly ($p > 0.05$) inhibit Gly-[^{3}H]L-Pro uptake into the Caco-2 cells, thus indicating that absorption is not *via* the bile acid transport system.

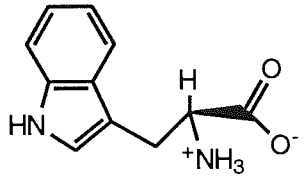
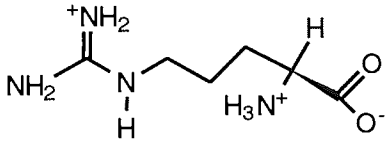
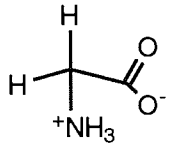
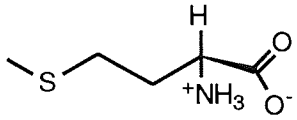
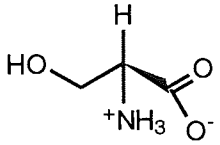
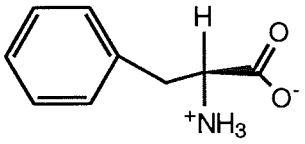
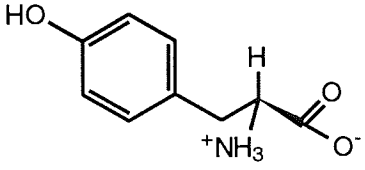
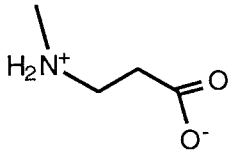
The effect of various amino acids on uptake was explored by comparing the uptake of Gly-[^{3}H]L-Pro alone or in the presence of 1 mM of the required amino acid. The amino acids L-Arg, Gly, L-Met, L-Phe, Sar, L-Ser and L-Tyr all failed to significantly inhibit uptake of Gly-[^{3}H]L-Pro into Caco-2 cells, thus ruling out that uptake occurs *via* these specific amino acid transport systems [Christensen, 1990]. The only compound tested which produced significant inhibition (18.92 ± 1.76) was the hydrophilic aromatic amino acid, L-Trp (Table 3.3), which may due to the fused ring system.

TABLE 3.2 *Gly-[^{3}H]L-Pro uptake in the presence of various compounds which have different methods of transport across the intestinal enterocytes*

Competitor	Structural Type	Transport Mechanism	% Inhibition Mean [S.D.]	IC_{50} mM Mean [S.D.]
Gly-[^{3}H]L-Pro (Control)	Dipeptide	H^+/di-/tripeptide system	0.00 [3.14]	
Bestatin	Dipeptide	H^+/di-/tripeptide system	67.38 [1.69] [a]	0.49 [0.04]
Glutathione	Tripeptide	Diffusion	25.04 [2.84] [b]	3.03 [0.49]
Gly-L-Pro-Gly-Gly	Tetrapeptide	Hydrolysis, absorption? Absorption *via* DTS?	41.68 [0.77] [a]	1.40 [0.04]
Taurocholic acid	Bile acid	Active transport *via* the bile acid carrier	-2.83 [4.26]	y
TRH	Tripeptide	Active transport? Passive transport ?	2.88 [4.89]	y
Sodium azide	Metabolic inhibitor		100.56 [0.75] [a]	x

Monolayers were washed (1 x 500 µl x 5 min) with M4. They were incubated with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6 or in the presence of 1 mM of the competitor. Data are corrected for 15 % non-specific uptake and presented as mean % inhibition ± S.D. and IC_{50} ± S.D. mM for three monolayers, with [a] and [b] donating significant inhibition at $p < 0.0001$ and $p < 0.001$ respectively. x and y correspond to inhibition values which are too large or too small (% inhibition $\geq$ 100.00, or $\leq$ 0.00 respectively) to be converted into a relevant IC_{50} value.

TABLE 3.3 *The effect of a series of amino acids on Gly-[^{3}H]L-Pro uptake*

Amino acid	Structure	Inhibition % Mean [S.D.]	IC_{50} mM Mean [S.D.]
L-tryptophan		18.92 [1.76][a]	4.32 [0.52]
L-arginine		13.37 [8.32]	y
glycine		3.43 [7.81]	y
L-methionine		3.12 [0.87]	33.15 [11.12]
L-serine		0.30 [3.24]	y
L-phenylalanine		-2.58 [6.24]	y
L-tyrosine		-5.52 [1.21]	y
Sarcosine		-12.05 [8.47]	y

Monolayers were washed (1 x 500 µl x 5 min) with M4, and then incubated with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6, or in the presence of 1 mM of amino acid. Data are corrected for 15 % non-specific uptake and presented as mean % inhibition ± S.D. for three monolayers and mean IC_{50} ± S.D. mM, with [a] denoting significant inhibition at $p< 0.05$. from the control (uptake in the absence of competitor). y corresponds to an inhibition value which is too small (% inhibition ≤ 0.00) to be converted into a relevant IC_{50} value.

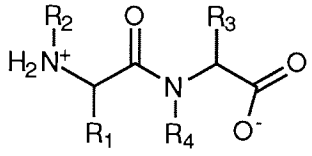
The ability of our method to rank the affinity of compounds for interaction with the DTS was investigated by co-administration of a series of dipeptides, at 1 mM, with the probe. All of the compounds tested showed a significant reduction in Gly-[^{3}H]L-Pro uptake (Table 3.4.) with the inhibition varying from 103.56 ± 2.08 (total inhibition of the active component) to 22.8 ± 4.53 % and the IC_{50} values corresponding to 0.10 ± 0.02 to 3.51 ± 0.97 mM. These data, confirm that the Gly-[^{3}H]L-Pro is being taken up into the cell *via* the DTS, and providing a system where structure-activity relationships of the DTS could be evaluated with the possibility of identifying the optimum structure for interaction. In this work, the competitors used to explore the specificity of Gly-L-Pro uptake and the dipeptides were used at 1 mM concentration (closer to the K_m values). This is in contrast to previous studies of this nature, which have used higher concentrations of compound where inhibition has been virtually complete. However, our intention was to obtain a range of inhibition values without saturating the interaction, by using a lower concentration of competitor, which would then better allow exploration of the impact of structural variations.

All the dipeptides tested produced significant inhibition of the probe with IC_{50} values ranging from 0.128 ± 0.018 mM to 3.51 ± 0.965 mM, suggesting that they all share the same dipeptide carrier. The dipeptide L-Phe-Gly inhibited all the active uptake of Gly-[^{3}H]L-Pro, supporting the view that a lipophilic side-chain at the *N*-terminus enhances transport [Matthews, 1987]. L-Ala-L-Val produced 90.51 ± 1.24 % inhibition; interestingly both amino acid components in this dipeptide have hydrophilic properties. Gly-DL-Met, Gly-DL-Phe and Gly-L-Pro all caused similar degrees of inhibition of Gly-[^{3}H]L-Pro uptake (85.2 - 88.7 %). One would expect 1 mM Gly-L-Pro to inhibit all the active uptake of the probe as the concentration used was in excess of the reported K_m value, but 85 % inhibition was achieved at 1 mM, suggesting that the expression of the transporter may differ between passage number and experimental days. However, the effect (inhibition) of SQ-29852 on the uptake of Gly-[^{3}H]L-Pro was consistent as shown in Table 3.1. The results for Gly-DL-Met (88.7 ± 1.39) and Gly-DL-Phe (86.1 ± 2.38) were not significantly different from each other. However, there was a significant ($p < 0.05$) difference between the inhibition from Gly-DL-Met and Gly-L-Pro (85.2 ± 1.31), thus suggesting that Gly-DL-Met displays the greatest affinity for the transporter. Gly-Sar had less affinity for the transporter than Gly-DL-Phe and Gly-L-Pro when co-administered with Gly-[^{3}H]L-Pro in human intestinal BBMV [Rajendran *et al.*, 1985a]. Our results were in agreement with this, and the same rank order of inhibition was observed as previously reported [Rajendran *et al.*, 1985a]. However, the difference between the activity of the Gly-L-Phe and Gly-L-Pro compared to that of Gly-Sar is much greater in our model than found in the human BBMV. L-Carnosine, which has been reported be inhibit transport of Gly-Sar [Thwaites *et al,* 1994a], and the dipeptides, Gly-L-Trp, L-Pro-L-Phe, Gly-L-Tyr and L-Phe-L-Pro all inhibited uptake of the probe. Sar-L-Pro which lacks a primary *N*-terminal α-amino group produced inhibition of the Gly-[^{3}H]L-Pro, but to a lesser extent (IC_{50} = 3.51 ± 0.97 mM) than the other compounds tested which all possess this structural feature, agreeing with previous literature that a free

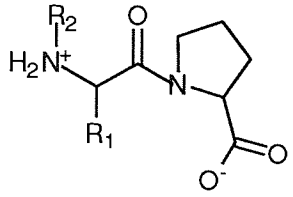
α-amino group is not essential for transport [Bai *et al.*, 1991]. The diacidic dipeptides, L-Asp-L-Asp and L-Glu-L-Glu, which have a net negative charge both caused inhibition of Gly-[^{3}H]L-Pro uptake, however to a lesser extent than the zwitterionic dipeptides, agreeing with a report that states that neutral dipeptides have a greater affinity for the transporter than di-anionic dipeptides [Eddy *et al.*, 1995]. This contradicts a previous report which states that there is no difference in interaction between linear dipeptides, regardless of charge, and the transporter [Hidalgo *et al.*, 1995] . However, the study in question used a different radiolabel ([^{3}H]cephalexin) and Caco-2 cells at a higher passage number (60-70). The competitors were used at a concentration of 10-20 mM which may saturate inhibition and mask the differential activity of dipeptides, also high concentrations may have a non-specific affect on the cells.

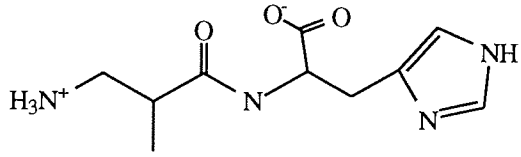
TABLE 3.4 *The effect of a series of dipeptides on Gly-[^{3}H]L-Pro uptake*

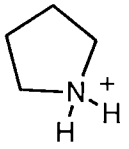
Structure A



Structure B



Dipeptide		R_1	R_2	R_3	R_4	Inhibition % Mean [S.D.]	IC_{50} mM Mean [S.D.]
L-Phe-Gly	A	$CH_2C_6H_6$	H	H	H	103.56[2.08]^a	x
L-Ala-L-Val	A	CH_3	H	$CH(CH_3)_2$	H	90.51 [1.24]^a	0.10 [0.02]
Gly-DL-Met	A	H	H	$(CH_2)_2SCH_3$	H	88.66 [1.39]^a	0.13 [0.02]
Gly-DL-Phe	A	H	H	$CH_2C_6H_6$	H	86.11 [2.38]^a	0.16 [0.03]
Gly-L-Pro	B	H	H	-	-	85.18 [1.31]^a	0.17 [0.02]
Gly-Sar	A	H	H	H	CH_3	72.13 [1.65]^a	0.39 [0.03]
L-carnosine						59.11 [1.95]^a	0.69 [0.09]
Gly-L-Trp	A	H	H	CH_2 indole	H	53.14 [1.42]^a	0.88 [0.05]
L-Pro-L-Phe	A	---CH_2--CH_2---CH_2--*		$CH_2C_6H_6$	H	51.46 [0.90]^c	0.94 [0.03]
L-Asp-L-Asp	A	CH_2COO^-	H	CH_2COO^-	H	45.83 [4.02]^b	1.19 [0.20]
Gly-L-Tyr	A	H	H	$CH_2C_6H_5OH$	H	41.37 [3.12]^a	1.43 [0.18]
L-Phe-L-Pro	B	$CH_2C_6H_6$	H	-	-	36.77 [1.29]^b	1.72 [0.09]
L-Glu-L-Glu	A	$(CH_2)_2COO^-$	H	$(CH_2)_2COO^-$	H	36.05 [2.00]^b	1.78 [0.15]
Sar-L-Pro	B	H	CH_3	-	-	22.81 [4.53]^c	3.51 [0.97]



* R_1 and R_2 fused to form .

Monolayers were washed (1 x 500 µl x 5 min) with M4, and then incubated with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6, or in the presence of 1 mM of dipeptide. Data are corrected for 15 % non-specific uptake and presented as mean % inhibition ± S.D. for three monolayers and mean IC_{50} ± S.D. mM, with ^{a}, ^{b} and ^{c} denoting significant inhibition at $p< 0.0001$, $p< 0.001$ and $p< 0.01$ respectively, in % inhibition from the control (uptake in the absence of competitor). x corresponds to an inhibition value which is too large (% inhibition ≥ 100.00) to be converted into a relevant IC_{50} value.

3.3.3 GLY-[^{3}H]L-PRO TRANSPORT ACROSS CACO-2 CELL MONOLAYERS

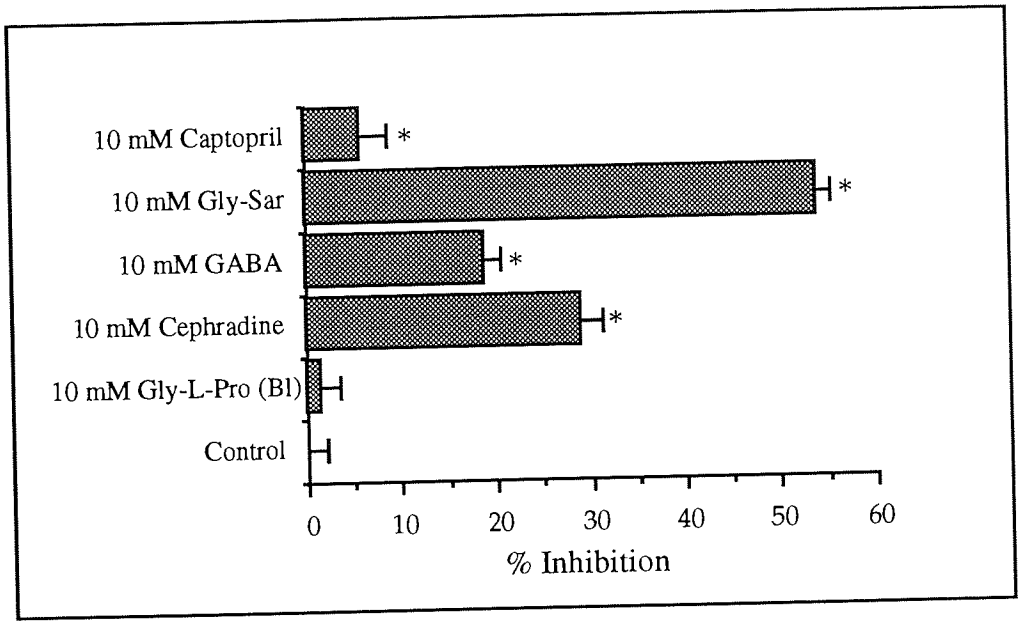
Mannitol flux across the monolayers was used as an indication of the formation of tight junction and therefore differentiation of the cell monolayers. Monolayers were discarded if mannitol flux was > 0.5 % of total mannitol present. The data was not expressed as permeability coefficients (P_{app}), as this parameter used for permeability of passively absorbed compounds and does not take into account the contribution from active processes, and therefore not appropriate for the Gly-L-Pro, which is actively absorbed *via* the DTS.

Total transport across Caco-2 cell monolayers for a 50 nM apical donor solution (apical pH 6.0-to-basolateral pH 7.4) for Gly-[^{3}H]L-Pro was 26.78 ± 0.60 pmol h^{-1} $insert^{-1}$ and for [^{3}H]L-Pro was 15.60 ± 1.09 pmol h^{-1} $insert^{-1}$. Gly-Sar, GABA and cephradine all produced a significant reduction (53.93 ± 1.57, 18.73 ± 1.92, and 28.95 ± 2.42 % inhibition respectively) in Gly-[^{3}H]L-Pro transport when co-administered with the control (Figure 3.15). Gly-Sar produced greater inhibition than cephradine which is surprising, considering that the reported K_m value in Caco-2 cells for Gly-Sar is 17.4 ± 5.1 mM [Thwaites *et al.*, 1993g] and that for cephardine is 8.3 mM [Inui *et al.*, 1992]. Cephradine, having a greater affinity for the transporter, would be expected to produce the greatest inhibition, but our model suggests that Gly-Sar has a greater affinity for the DTS, in the present system. However, Thwaites *et al.* used Caco-2 cells at passage number 119-121, which may explain the apparent difference in affinities.

Much controversy and contradictory literature surrounds the absorption characteristics of captopril. The present study showed that captopril produced a slight but significant inhibition on Gly-[^{3}H]L-Pro transport. This is in agreement with a recent report stating that captopril inhibits Gly-Sar transport across Caco-2 cell monolayers [Thwaites *et al.*, 1995]. Captopril has also been shown to bind to protein [Wong *et al.*, 1981] and therefore its inhibitory effect may be due binding to the transporter protein in the DTS and blocking uptake of the probe or non-specifically binding to membrane proteins leading to a decrease in free captopril available too inhibit the probe. 10 mM Gly-L-Pro in the receiver phase (basolateral compartment) had no effect on Gly-[^{3}H]L-Pro transport, indicating that the transporter can work against an uphill concentration gradient indicating an active mechanism.

FIGURE 3.15 *The effect of various competitors on the transport of Gly-[^{3}H]L-Pro across Caco-2 monolayers*

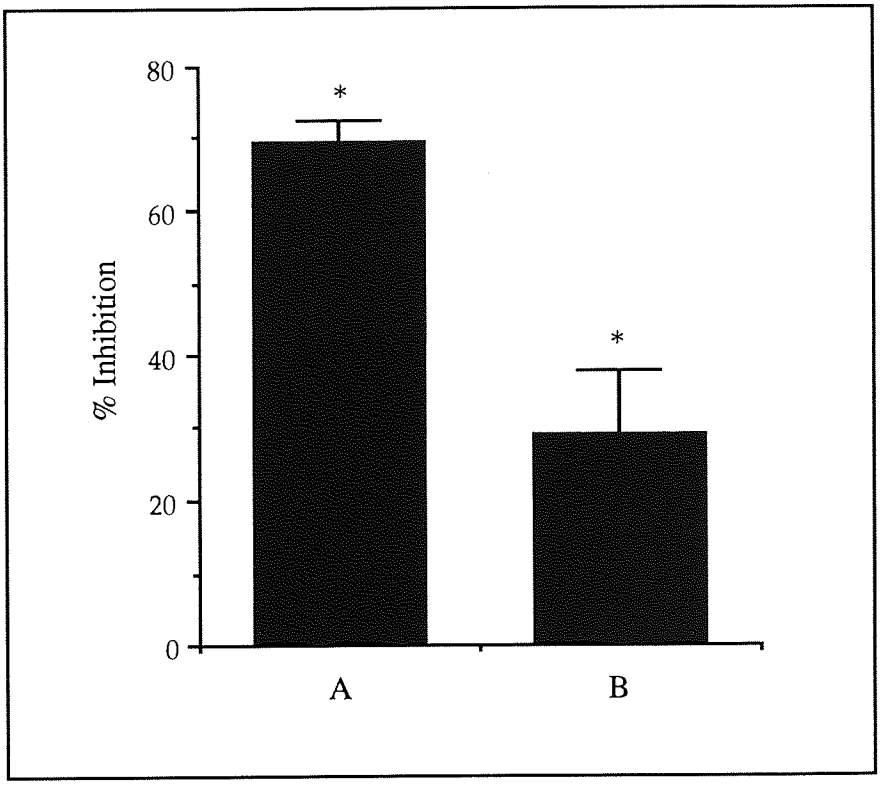
The monolayers were washed [1 x (1.5 ml apical) + (2.6 ml basolateral) x 15 min] at 37°C with M5 at pH 7.4, and then incubated [1 x (1.5 ml apical)] with M4 at pH 6, containing 50 nM unlabelled Gly-L-Pro and 8 nM Gly-[^{3}H]L-Pro in the presence of competitor. Inserts were placed in M5 at pH 7.4 (2.6 ml basolateral phase) and incubated for 30 min, and then transferred to a fresh basolateral phase for a further 30 min. The effect of a concentration gradient on transport was investigated by having 10 mM Gly-L-Pro in the basolateral phase. Data are mean (of at least three monolayers) ± S.D. and expressed as % inhibition from control (Gly-[^{3}H]L-Pro transport in the receiver phase). * Denotes a significant ($p < 0.05$) reduction from the control value.



The majority of Gly-[^{3}H]L-Pro will be broken down into its component amino acids intracellulary by the cytosolic enzyme prolidase, and then the amino acids will be transported across the basolateral membrane. Therefore, in effect, we are measuring [^{3}H]L-Pro content in the basolateral phase. However, enzymatic hydrolysis at the apical brush border membrane may occur as illustrated by previous results (Figure 3.4 B), leading to production of [^{3}H]L-Pro, which will be transported across the monolayers resulting in an elevated tritium count for the basolateral compartment and giving an over-estimation of dipeptide transport. In order to achieve an accurate estimation for Gly-[^{3}H]L-Pro transport, [^{3}H]L-Pro uptake at the apical membrane needs to be suppressed/inhibited. The imino-acid carrier system in Caco-2 cells has been reported to be concentration- and Na^+-dependent [Nicklin *et al.*, 1992a]. Therefore, the effect of excess unlabelled L-Pro and the absence of Na^+ from the incubation media, on the transport of Gly-[^{3}H]L-Pro and [^{3}H]L-Pro were evaluated. 10 mM L-Pro in the apical phase produced a significant reduction in [^{3}H]L-Pro and Gly-[^{3}H]L-Pro and transport across Caco-2 cell monolayers, producing 69.34 ± 3.03 and 29.05 ± 8.66 %, inhibition respectively (Figure 3.16). Excess L-Pro seems to be having a greater effect on Gly-[^{3}H]L-Pro transport than uptake, 29.05 ± 8.66 % compared to 6.68 ± 2.38 % (USA result) respectively. Note that the transport studies were carried out in the USA laboratories, thus explaining the reason for the above comparison. This may possibly be explained by the L-Pro being taken up into the cells *via* the imino acid transporter and competing with the Na^+-dependent system A transporter on the basolateral membrane which is responsible for efflux of [^{3}H]L-Pro into the receiver compartment [Stevens *et al.*, 1984: Barker *et al.*, 1990]. Another route of action may occur intracellularly after the L-Pro has penetrated the cell membrane [Nicklin *et al.*, 1992a]. L-Pro will inhibit prolidase activity (66 %) [Hu *et al.*, 1994a], resulting in partial hydrolysis of the probe. It has been suggested that the basolateral DTS is underexpressed in Caco-2 cells [Chong *et al.*, 1996] and therefore effects the efflux of the unhydrolysed probe, resulting in an apparent inhibition of transport. [^{3}H]L-Pro may also be effluxed from the cell into the apical compartment again leading to an apparent inhibition of transport.

FIGURE 3.16 *The effect of excess L-Pro on the transport of [^{3}H]L-Pro and Gly-[^{3}H]L-Pro across Caco-2 cell monolayers.*

The monolayers were washed [1 x (1.5 ml apical) + (2.6 ml basolateral) x 15 min] at 37°C with M5 at pH 7.4, and then incubated [1 x (1.5 ml apical)] with M4 at pH 6, containing A) 50 nM unlabelled L-Pro and 4 nM[^{3}H]L-Pro or B) 50 nM unlabelled Gly-L-Pro and 4 nM Gly-[^{3}H]L-Pro. Inserts were placed in M5 at pH 7.4 (2.6 ml basolateral phase) and incubated for 30 min, and then transferred to a fresh basolateral phase for a further 30 min. The effect of excess L-Pro on transport was investigated by coadministering A and B with 10 mM L-Pro.. Data are mean (of at least three monolayers) ± S.D. and expressed as % inhibition from control ([^{3}H]L-Pro [**A**] or Gly-[^{3}H]L-Pro [**B**] transport into the receiver phase in the absence of L-Pro). * Denotes a significant ($p < 0.05$) reduction from control.



L-Pro uptake was found originally to have a high degree of Na^+-dependence [Nicklin *et al.*, 1992a] and, therefore as dipeptide uptake is Na^+-independent, the absence of Na^+ from the media should suppress uptake of [^{3}H]L-Pro, without affecting Gly-[^{3}H]L-Pro transport. The absence of Na^+ from the transport medium had a significant effect on both Gly-[^{3}H]L-Pro and [^{3}H]L-Pro transport across Caco-2 cell monolayers, reducing transport by 36.35 ± 3.28 % and 21.28 ± 8.0 % respectively (Figure 3.17), indicating that Na^+ plays a role in both transport processes. The inhibition of Gly-[^{3}H]L-Pro transport in the absence of Na^+ could suggest that the probe is being hydrolysed at the apical surface and the resulting [^{3}H]L-Pro can not be taken up into the cell due to the lack of Na^+. However, this can be ruled out as [^{3}H]L-Pro transport was only inhibited by 21.28 ± 8.0 % in the absence of Na^+, indicating a Na^+-independent system. This is in agreement with Thwaites *et al.* who claim that Caco-2 cells possess an apically-localised, Na^+-independent, electrogenic H^+/imino acid transporter [Thwaites *et al.*, 1993f]. The 21.08 ± 8.0 % inhibition can be attributed to the suppression of the Na^+-dependent system A transporter at the basolateral membrane [Stevens *et al.*, 1984: Barker *et al.*, 1990]. The driving force behind the DTS is *via* a H^+/cotransport system, with the electrochemical gradient for H^+ energising absorption, rather than Na^+. However, Na^+ has been suggested to play an indirect role in DTS transport by assisting in the generation and maintenance of the H^+ gradient through the Na^+-H^+-exchanger located at the brush-border membrane and the Na^+-K^+-ATPase pump at the basolateral membrane [Hoinard *et al.*, 1988]. These results indicate that Na^+ plays a substantial role in dipeptide absorption even if only *via* an indirect route.

The ACE-inhibitor SQ-29852 produced a significant reduction in Gly-[^{3}H]L-Pro transport which was concentration-dependent, 77.86 ± 0.96 % and 37.03 ± 4.76 % at 10 mM and 1 mM respectively (Figure 3.18). Neither the D- or the L- form of captopril had a significant effect on Gly-[^{3}H]L-Pro transport. However, previously we have shown that 10 mM has a slight but significant effect on transport and therefore can conclude that the concentration used in this experiment is not high enough, and that the K_m for captopril exceeds 1 mM.

FIGURE 3.17 *The effect of Na^+ on the transport of [^{3}H]L-Pro and Gly-[^{3}H]L-Pro across Caco-2 cell monolayers*

The monolayers were washed [1 x (1.5 ml Apical) + (2.6 ml Basolateral) x 15 min] at 37°C with M5 at pH 7.4, and then incubated [1 x (1.5 ml Apical)] with M4 at pH 6, containing A) 50 nM unlabelled L-Pro and 4 nM[^{3}H]L-Pro or B) 50 nM unlabelled Gly-L-Pro and 4 nM Gly-[^{3}H]L-Pro. Inserts were placed in M5 at pH 7.4 (2.6 ml basolateral phase) and incubated for 30 min, and then transferred to a fresh basolateral phase for a further 30 min. The effect of Na^+ on transport was investigated by repeating A and B in Na^+-free M4. Data are mean (of at least three monolayers) ± S.D. and expressed as % inhibition from control ([^{3}H]L-Pro [**A**] or Gly-[^{3}H]L-Pro [**B**] transport into the receiver phase in the presence of Na^+). * Denotes a significant ($p < 0.05$) reduction from control.

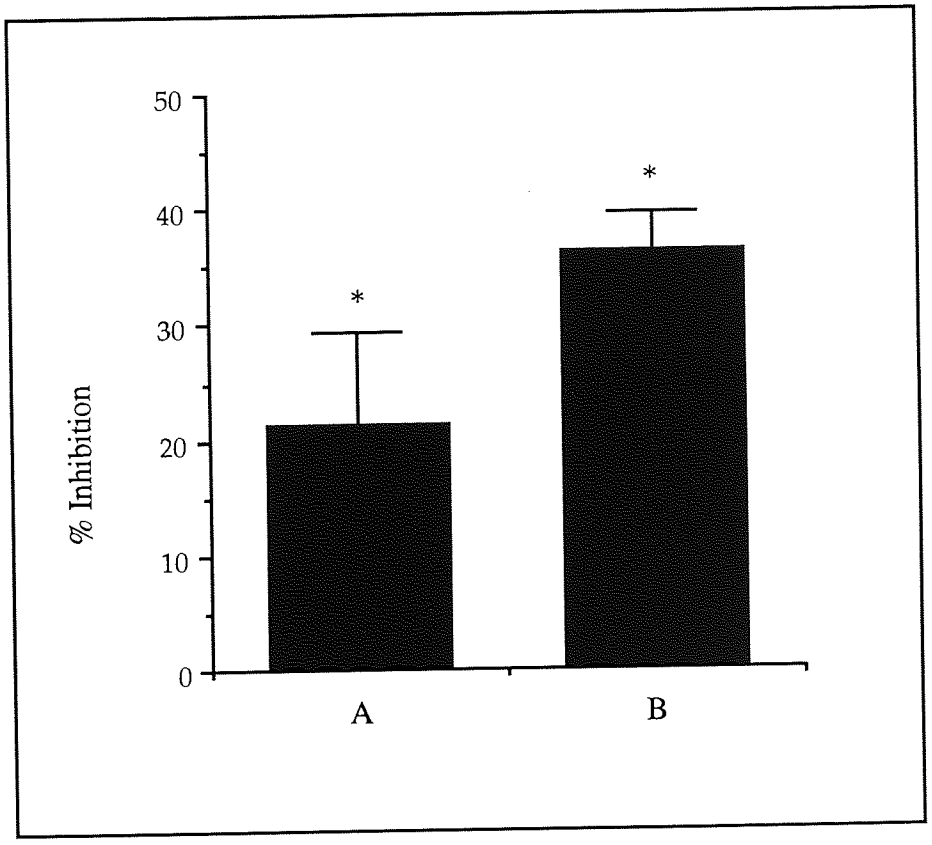
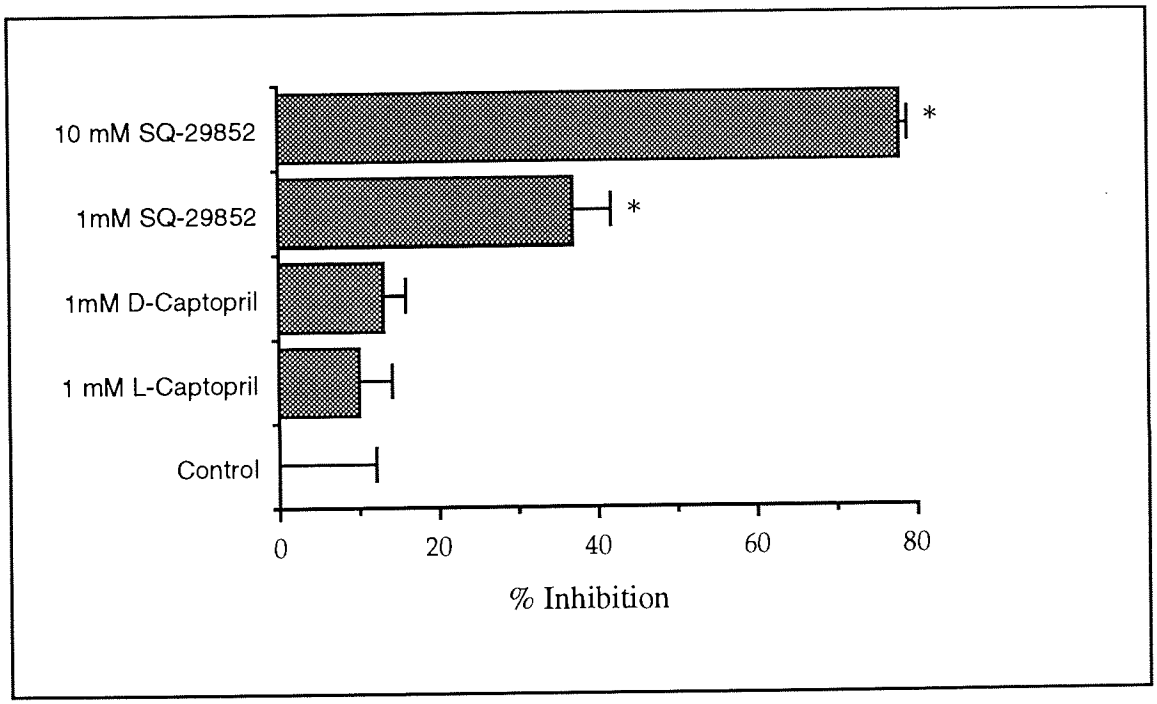


FIGURE 3.18 *The effect of the ACE-inhibitors, Captopril and SQ-29852, on the transport of Gly-[^{3}H]L-Pro across Caco-2 monolayers*

The monolayers were washed [1 x (1.5 ml Apical) + (2.6 ml Basolateral) x 15 min] at 37°C with M5 at pH 7.4, and then incubated [1 x (1.5 ml Apical)] with M4 at pH 6, containing 50 nM unlabelled Gly-L-Pro and 4 nM Gly-[^{3}H]L-Pro in the presence of competitor. Inserts were placed in M5 at pH 7.4 (2.6 ml basolateral phase) and incubated for 30 min, and then transferred to a fresh basolateral phase for a further 30 min. Data are mean (of at least three monolayers) ± S.D. and expressed as % inhibition from control (Gly-[^{3}H]L-Pro transport into the receiver phase in the absence of competitor). * Denotes a significant ($p < 0.05$) reduction from the control value.



3.3.4 TRIPEPTIDE STABILITY IN THE PRESENCE OF CACO-2 CELL MONOLAYERS

The tripeptide L-Phe-Gly-Gly was broken down when incubated with Caco-2 cell monolayers (Figure 3.19 A). After a 90 min period all the 1 mM tripeptide had been hydrolysed producing 1 mM L-Phe, indicating that there was no significant loss of L-Phe by absorption into the cell monolayer and no L-Phe-Gly was produced during the degradation process. Therefore, the reaction followed the scheme shown below;

L-Phe-Gly-Gly → **ENZYMATIC DEGRADTION** → L-Phe + Gly-Gly

↓

2 Gly

Enzyme reactions follow the Michaelis-Menten kinetics and can be fitted to the following model shown in Equation 3.5 [Irwin *et al.*, 1995]:

EQUATION 3.5

$$\ln (S_t) + \frac{S_t}{K_m} = \ln (S_0) + \frac{S_0}{K_m} - \frac{V_{max}}{K_m}.t$$

Where S_0 is the initial substrate concentration and S_t represents its value at time t. The above model approximates to zero-order when $S_0 >> K_m$, and to first order when $K_m >> S_0$, with a first-order rate constant of V_{max} / K_m. The reaction profile of L-Phe-Gly-Gly breakdown was first-order, and thus, the following equation applied (Equation 3.6);

EQUATION 3.6

when $K_m >> S_0$

$$\ln S_0 >> \frac{S_0}{K_m}$$

so,

$$\ln S_t = \ln S_0 - \frac{V_{max}}{K_m}.t$$

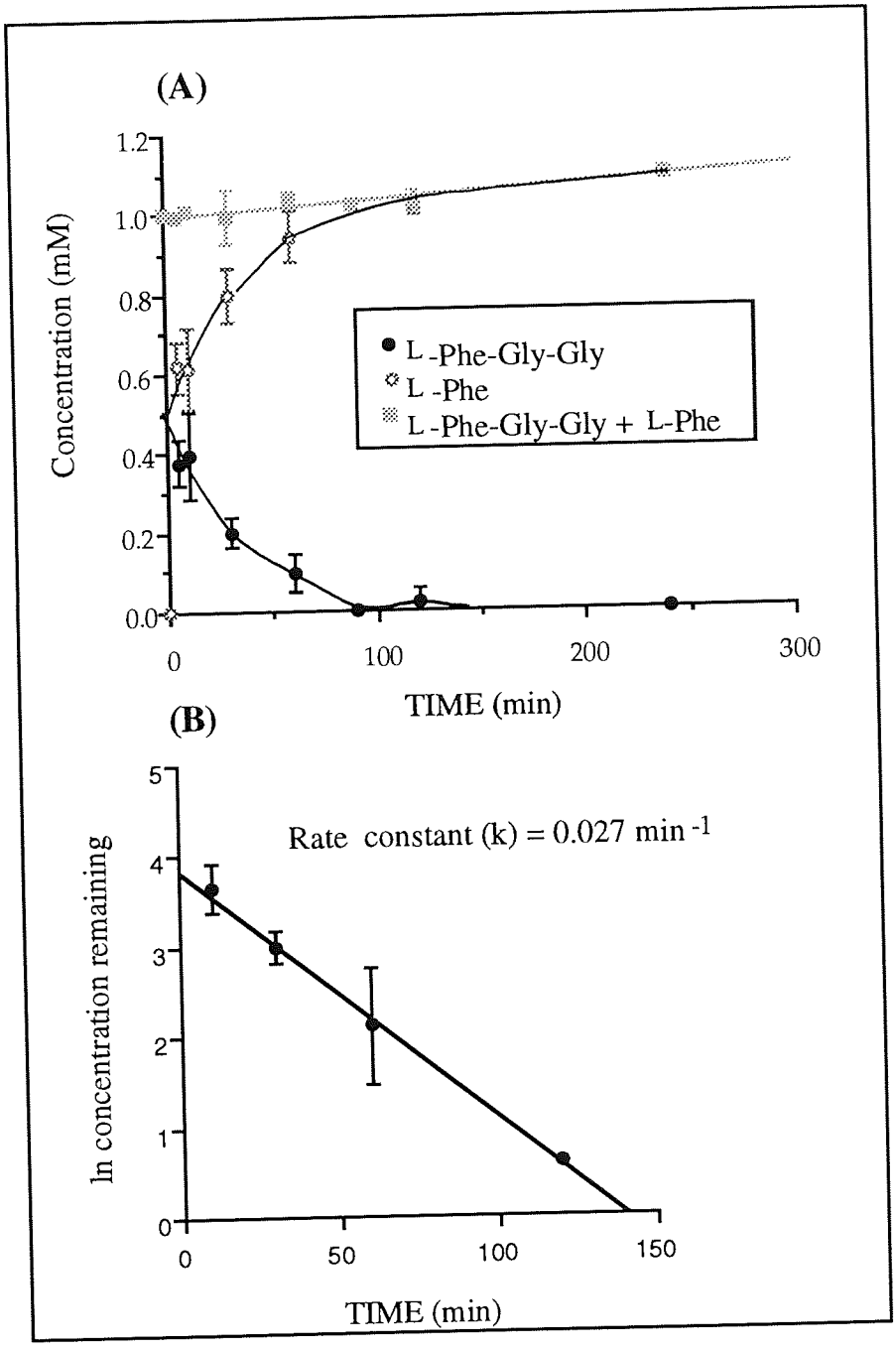
where the first order rate constant (k) is:

$$k = \frac{V_{max}}{K_m}$$

The first-order rate constant (k) for tripeptide breakdown was 0.027 min^{-1}, which was calculated by plotting the ln (concentration remaining) against time (Figure 3.19 B). It is expected that L-Phe-Gly-Gly will be broken down at the brush-border membrane rather than cytosolically as the brush-border peptidases have a preference for tripeptides, especially when the *N*-terminus residue has a lipophilic side chains, such as L-Phe [Matthews & Payne, 1980]. Brush-border peptidases have also been shown to play an important role in the mucosal hydrolysis of L-Phe-Gly [Silk *et al.*, 1976]. The tripeptide is being cleaved at the peptide bond between the L-Phe and Gly, as no L-Phe-Gly was detected in the apical medium and the L-Phe recovered was proportional to the tripeptide hydrolysed. It is also unlikely that enzymatic hydrolysis is occurring between Gly-Gly as no hydrolysis of this dipeptide was detected by membrane bound enzymes in a previous study [Adibi, 1971].

FIGURE 3.19 *Breakdown of L-Phe-Gly-Gly when incubated with Caco-2 cell monolayers*

The monolayers were washed (1 x 2 ml x 30 min) with M5 at pH 7.4, and then incubated with a 1 mM L-Phe-Gly-Gly solution. Breakdown of the tripeptide and appearance of L-Phe were monitored at the following time points; 5, 10, 30, 60, 90, 120 and 240 min. Data are expressed as mean (of at least three monolayers) ± S.D. for **(A)** (concentration at a given time). Rate of breakdown of L-Phe-Gly-Gly was calculated from these data **(B)**.



L-Phe-Gly-Gly incubated with Caco-2 cell monolayers of increasing age (4, 7, 10, and 21 d post-seeding) (Table 3.5) illustrated that enzymatic expression is effected by cell age as previously suggested [Beaulieu & Quaroni, 1991: Darmoul *et al.*, 1992]. Breakdown with 4 d old cells was extremely rapid, with no L-Phe-Gly-Gly remaining at the first time point (5 min). 7, 10 and 21 d old cells all produced similar break down profiles, however at 120 min there was still some L-Phe-Gly-Gly present, which disagrees with the previous result (Figure 3.19a), suggesting that enzymatic expression may well differ with passage number. The rate constant values for breakdown of L-Phe-Gly-Gly after 7, 10 and 21 d in culture, were similar with values of 0.025 min^{-1}, 0.022 min^{-1} and 0.022 min^{-1} respectively.

TABLE 3.5 *Breakdown of L-Phe-Gly-Gly when incubated with Caco-2 cell monolayers of increasing age (4 - 21 d).*

	% L-Phe-Gly-Gly remaining Mean [S.D.]			
TIME (min)	DAY 4	DAY 7	DAY 10	DAY 21
0	100	100	100	100
5	0	37.65 [5.91]	41.11 [7.96]	61.02 [4.04]
15	0	26.50 [6.70]	22.62 [7.12]	32.10 [1.02]
30	0	19.99 [3.77]	13.51 [6.39]	14.72 [4.96]
60	0	9.41 [4.81]	5.91 [2.47]	12.11 [2.06]
120	0	2.09 [0.61]	2.86 [0.98]	3.71 [1.29]
240	0	0	0	0
360	0	0	0	0
R^2	-	0.999	0.992	0.911
k	-	0.025 min^{-1}	0.022 min^{-1}	0.022min^{-1}

The monolayers were washed (1 x 2 ml x 30 min) with M5 at pH 7.4, and then incubated with a 1 mM L-Phe-Gly-Gly solution. Breakdown of the tripeptide and appearance of L-Phe were monitored at the following time points; 5, 15, 30, 60, 90, 120, 240 and 360 min. The experiment was carried out using cells after 4, 7, 10 and 21 d post-seeding. Data are expressed as mean (of at least three monolayers) ± S.D. Rate of breakdown of L-Phe-Gly-Gly was calculated from the data, for each experimental d, with R^2 and the rate constant (k) shown in Table 3.5. Note that for d 4 all the tripeptide was hydrolysed by the first time point (5 min) and therefore the rate constant was not calculated.

To assess whether the enzymes responsible for L-Phe-Gly-Gly breakdown were membrane bound or released extracellularly into the apical solution, the tripeptide (0.5 mM) was incubated with M5 that had been in contact with Caco-2 cells for 10, 30 and 60 min. Figure 3.20 shows that L-Phe-Gly-Gly breakdown occurs with the apical solutions, suggesting that enzyme is released from the cells into the environment. The apical solution which had been in contact with the cells for 60 min produced the greatest breakdown of L-Phe-Gly-Gly, suggesting the release of enzyme is time-dependent. After 60 min there was still 0.15 ± 0.05 mM L-Phe-Gly-Gly remaining. L-Phe-Gly-Gly breakdown therefore is likely to be a combination of enzymes released into the apical solution and membrane bound enzymes. However, cell shedding into the apical solution may also be a contributing factor to hydrolysis that needs considering.

Caco-2 cells have been reported to express a LNAA carrier, which is responsible for the active uptake of L-Phe [Hidalgo & Borchardt, 1990a: Hu & Borchardt, 1992]. However, the present study found that as L-Phe-Gly-Gly was broken down L-Phe appeared at the same concentration, suggesting that L-Phe is not taken up into the cells. However, Figure 3.21 shows that 8.01 ± 0.25 μM of L-Phe was taken up into the cells over a 6 hr period. The HPLC method used, was not sensitive enough to detect the decrease in L-Phe concentration (8.01 ± 0.25 μM) in the apical phase and therefore gives the impression that L-Phe is not transported into the cells.

FIGURE 3.20 *Breakdown of L-Phe-Gly-Gly when incubated with apical solutions from Caco-2 cell monolayers*

The monolayers were washed (1 x 2 ml x 30 min) with M5 at pH 7.4, and then incubated with a M5 at pH 7.4 (1 x 2 ml), for the following time points; 10, 30 and 60 min. Apical solutions were removed, added to a 1 mM solution of L-Phe-Gly-Gly and incubated at 37°C for 60 min. Breakdown of the tripeptide and appearance of L-Phe were monitored. Data are expressed as mean (of at least three monolayers) ± S.D.

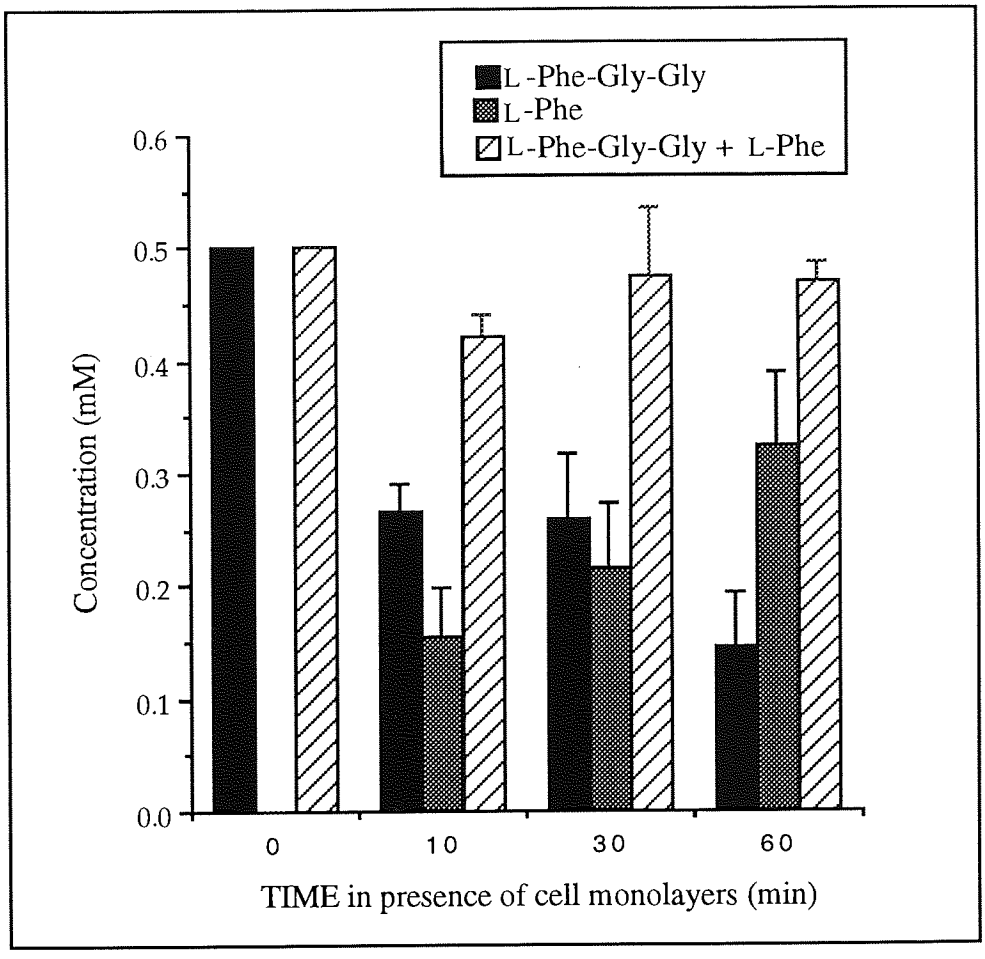
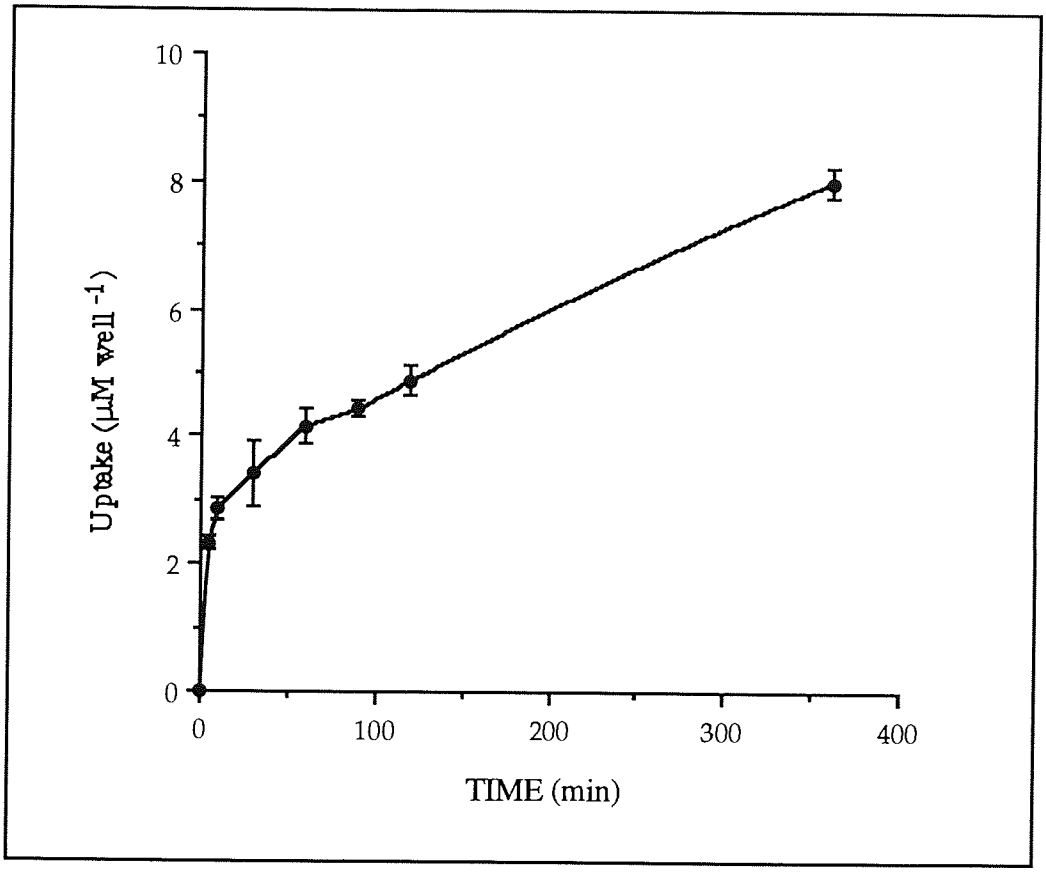


FIGURE 3.21 *Uptake of [^{3}H]L-Phe into Caco-2 cell monolayers*

The monolayers were washed (1 x 2 ml x 30 min) at 37°C, with M5 at pH 7.4, and then incubated with a M5 at pH 7.4 containing 5.4 nM [^{3}H]L-Phe and 1 mM unlabelled L-Phe. Uptake into the cells was monitored after the following time points; 5, 10, 30, 60, 90, 120, 240 and 360 min. Data are expressed as mean (of at least three monolayers) ± S.D.



3.4 CONCLUSION

In conclusion, Gly-[^{3}H]L-Pro is taken up into Caco-2 cell monolayers by a major saturable (carrier-mediated, approximately 85 % at 5 mM) pathway superimposed onto a minor non-saturable (diffusional) pathway. The process was found to be concentration-, pH- and temperature-dependent. Uptake was inhibited by small peptides and SQ-29852 indicating that the DTS is the system utilised. Some hydrolysis of the probe occurred at the brush border membrane, with cells in the UK laboratory producing greater hydrolysis than those in at the USA laboratory, indicating differences between laboratories and cell clones. Gly-[^{3}H]L-Pro transport across Caco-2 cell monolayers was mediated *via* the DTS, with the dipeptide Gly-Sar, GABA, cephradine and captopril all producing inhibition of transport.

The tripeptide L-Phe-Gly-Gly which was cleaved at the bond between the L-Phe and Gly, was hydrolysed completely in the apical compartment. Enzyme activity was found to be present in the apical media suggesting that the enzyme responsible for hydrolysis is released extracellularly into the surrounding environment, or cell shedding may be occurring.

The present system has been designed and validated for use in the investigation of the structural requirements of the DTS. Inhibition by a series of dipeptides illustrated that at low concentrations (1 mM) relevant affinities for the transporter can be ranked without saturating the system, allowing the true interaction to be interpreted.

CHAPTER 4

THE EFFECT OF SQ-29852 AND GLY-L-PRO ANALOGUES ON THE UPTAKE OF GLY-[^{3}H]L-PRO INTO CACO-2 CELL MONOLAYERS

ABSTRACT

Design of compounds having a high affinity for the DTS, as a potential enhancement of oral absorption, necessitates that the structural features for recognition be defined. A systematic approach was used to assess the structural features required for optimum interaction with the transporter. The DTS recognition sites were divided into domains A-F, based on the structure of SQ-29852, a stable and specific probe for the transporter [Marino *et al.*, 1996: Nicklin *et al.*, 1996]. 54 Gly-L-Pro and SQ-29852 analogues were divided into categories based upon the structural variations within specific regions, and their affinities for the transporter were measured by their ability to inhibit uptake of the probe Gly-[^{3}H]L-Pro, into Caco-2 cells.

Domain A illustrated a preference for a hydrophobic group, with a positive charge and H^+-acceptors and donor resulting in a loss of affinity for the DTS. SQ-29852 analogues were more tolerant of substitutions in the C domain compared to Gly-L-Pro analogues, with the preference being for a positive charge. The majority of substitutions in the D domain resulted in an increase in affinity for the DTS, with lipophilic aliphatic groups at position 4 on the proline ring and hydrophobic constrained groups in place of the proline ring being well accepted. SQ-30851 (dithiomethyl group at position 4) showed the greatest affinity for the transporter, illustrating a possible modification to oral drug candidates for increasing interaction with the DTS.

4.1 BACKGROUND

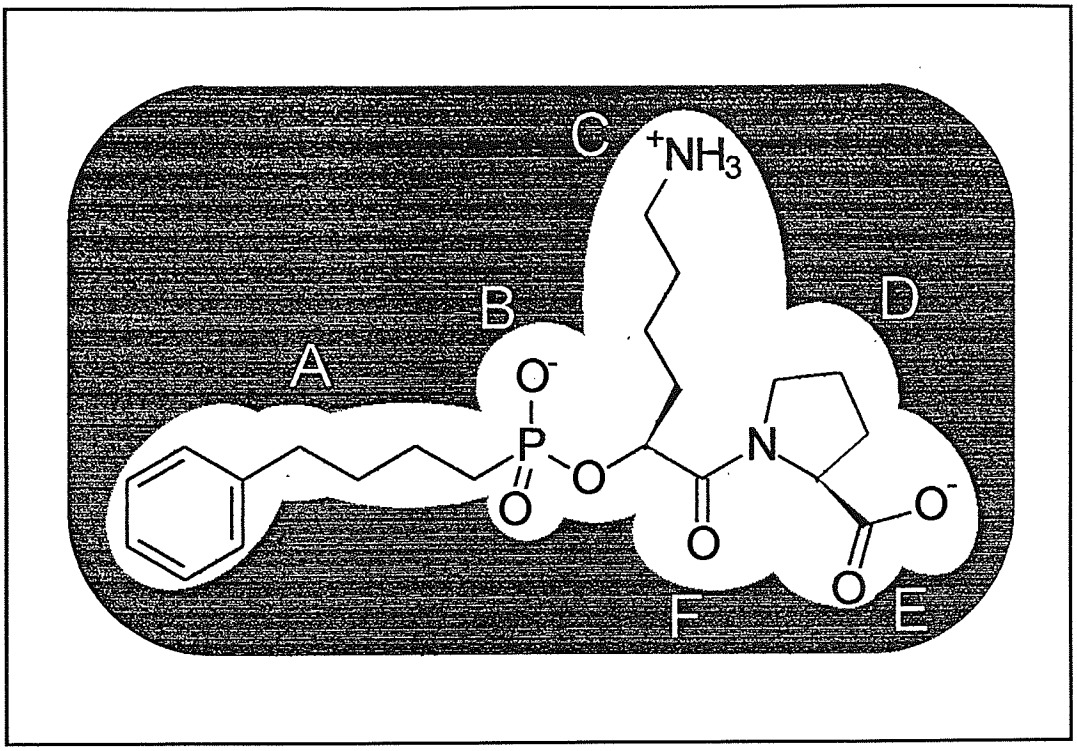
4.1.1 INTRODUCTION

An uptake model system has been designed and validated previously (Chapter 3), for use in investigating the structural requirements needed for substrate recognition by the DTS. Binding to the DTS is the first step in the process of translocation and is, therefore, important to the overall transport of molecules. The present model measures the inhibition of the control compound (uptake of Gly-[^{3}H]L-Pro in the presence and absence of competitors) and, therefore one must not assume that an inhibiting competitor is transported. Several studies have already demonstrated that compounds can bind to the peptide transporter without necessarily undergoing transport [Miyazaki *et al.*, 1982: Kramer *et al.*, 1992: Eddy *et al.*, 1995]. Therefore, the structural features which favour binding may be somewhat different to those that enhance transport. However, binding is the initial stage of the process, and a prerequisite to transport.

Information on the structural requirements needed for a molecule to bind to the DTS are limited. Previous competition studies have used a variety of probes (cephalosporins) and animal models (rat and rabbit BBMV, and the perfusion method) which are not directly comparable. Usually, only a handful of commercially available competitors are used within these studies, and therefore the information gained is somewhat sparse. Generally, a large concentration of competitor is used (10-20 mM), which may saturate the inhibition of the probe, swamping the system, and preventing the relative activity of competitors being assessed.

Some of the peptidomimetic ACE-inhibitors (*e.g.* lisinopril [Swaan *et al.*, 1995], quinapril [Hu *et al.*, 1995] and benazepril [Kim *et al.*, 1994]), have been found to have an active transport component *via* the DTS, which contributes to their oral absorption. The aim of the present chapter is to categorise the structural requirements needed for recognition by the DTS, using analogues of the dipeptide Gly-L-Pro, and the ACE-inhibitor, SQ-29852. As the work was carried out at Bristol-Myers Squibb, Princeton, USA, a large number (> 5000) of Gly-L-Pro and SQ-29852 analogues were available from the in-house library, thus enabling a systematic study to take place. The SQ-29852 structure was used as a model for interaction of ACE-inhibitors/competitors with the DTS, with the various areas of the structure being divided into domains A-F (Figure 4.1). The selection criteria of competitors were based on structural variations within specific regions of molecules, using the domains A-F (Figure 4.1).

FIGURE 4.1 *A model for the interaction of ACE-inhibitors with the intestinal DTS exemplified with SQ-29852. The illustrated domains are: A-F.*



4.1.2 SQ-29852 ABSORPTION MECHANISMS

SQ-29852 is a non-sulphydril phosphonic acid isostere of the dipeptide L-Lys-L-Pro, which has been found to have an oral bioavailability in humans of 67 % [Foley *et al.*, 1988], which is dose-dependent [Moore *et al.*, 1988]. SQ-29852 absorption in rat jejunum segments (perfusion method) was found to be concentration dependent and reduced in the presence of L-Tyr-Gly and cephradine, indicating an active, carrier-mediated process, *via* the DTS [Friedman & Amidon, 1989a]. A passive component responsible for approximately 15 % of absorption was also present. SQ-29852 has been shown to be a stable and specific probe for the DTS [Marino *et al.*, 1996]. Absorption along the intestinal tract of rats was dose-dependent, agreeing with the previous study where 85 % was absorbed *via* a saturable process. The poor passive permeability of SQ-29852 is due to its high charge at physiological pH, and its very low lipophilicity (Log P = -0.77) [Ranadive *et al.*, 1992]. This is in agreement with results obtained from an *in situ* rat intestinal perfusion model, where SQ-29852 absorption was found to be saturable and dose-dependent [Morrison *et al.*, 1996]. SQ-29852 absorption was found to occur throughout the GI tract with no significant difference in absorption between the various regions [Marino *et al.*, 1996].

4.1.3 SQ-29852 ABSORPTION IN CACO-2 CELL MONOLAYERS

Studies in this area are somewhat lacking, which is surprising considering the apparent suitability of SQ-29852 as a probe compound [Marino *et al.*, 1996]. Uptake into Caco-2 cell monolayers was found to be concentration-dependent over a 10 μM to 5 mM

range, comprising of a saturable and non-saturable process [Nicklin *et al.*, 1996]. 95 % of uptake was found to be due to an active process at ≤ 5 mM, and the K_m value was found to be higher than that previously reported for uptake into rat intestine (0.91 ± 0.11 mM in Caco-2 cells, compared to 0.08 mM for the rat) [Friedman & Amidon, 1989a: Nicklin *et al.*, 1996]. Although SQ-29852 absorption has been shown to be high, and almost exclusively *via* the DTS, the transport across Caco-2 cell monolayers was low, with a permeability coefficient less than that for PEG_{4000} (hydrophilic paracellular marker) [Nicklin *et al.*, 1996]. Transport was found to be non-saturable and whereas dipeptides inhibited uptake, no inhibition of transport was observed with the same compounds [Nicklin *et al.*, 1996]. Uptake into Caco-2 cell monolayers appears to be *via* a carrier-mediated process (DTS), but transepithelial transport is a passive mechanism, suggesting that the DTS is absent or under-expressed at the basolateral membrane [Nicklin *et al.*, 1996]. The recent findings with regard to SQ-29852 uptake and transport in Caco-2 cell monolayers [Nicklin *et al.*, 1996] highlight the appropriate selection of an uptake model over a transport model for the current studies.

4.1.4 STRUCTURAL REQUIREMENTS FOR THE DTS

Until now a systematic approach investigating the structural requirements of the DTS has not existed, resulting in an incomplete story, which is hard for chemists to translate into drug development projects. Most of the information available focuses on substitutions at the amino- and carboxy-terminals, ignoring the rest of the structure.

Best affinity is seen when both the amino- and carboxy-terminal are free. However, peptides with a terminal imino group, such as L-Phe-Gly, and those with an amidated carboxyl group are also transported, although with reduced affinity [Matthews, 1987]. Esterification or reduction to an alcohol of the carboxylic acid (Domain E) in L-Val-L-Val reduced activity by 50 %, illustrating that a free carboxy group is important for recognition [Hidalgo *et al.*, 1995]. α-Amino linkages are preferred although methylation of the N of peptide bonds (*e.g.* Gly-Sar) is tolerated. Cyclisation of certain dipeptides (*e.g.* Gly-L-Pro and L-Ala-L-Ala) resulted in a loss of affinity, however cyclised L-Glu-L-Glu and L-Asp-L-Asp retained their affinity, indicating the importance of dipeptide composition and the fact that conformationally constrained dipeptides are still substrates [Hidalgo *et al.*, 1995].

Originally it was thought that an *N*-terminal α-amino group was required for recognition [Rubino *et al.*, 1971], but recently competition studies using dipeptides lacking this structure have shown that these are transported, thus indicating that a free α-amino group in not essential for transport [Bai *et al.*, 1991]. This is not surprising, considering that several of the ACE-inhibitors and cephalosporins lacking the *N*-terminal α-amino group are reported to be transported *via* the DTS [Hu & Amidon, 1988: Friedman & Amidon 1989a, 1989b: Kramer *et al.*, 1990b: Gochoco *et al.*, 1994]. Substituting the *N*-terminal or *C*-terminal carboxyl groups has also been shown to reduce affinity [Addison *et al.*, 1975b]. The presence of lipophilic side chains, such as L-Phe, have also been reported to enhance

transport [Matthews, 1987]. Peptides containing basic or acidic amino acids have been shown to have a lower affinity for transport [Addison *et al.*, 1975b]. The DTS is tolerant of a single positive charge, with little change in affinity. However, the presence of a negative or a double positive charge greatly reduces affinity [Wootton & Hazelwood, 1989]. This is confirmed by another study that found dipeptides with a double positive charge exhibited the lowest affinity, while neutral dipeptides showed the greatest affinity [Eddy *et al.*, 1995].

Kramer has proposed a hypothetical peptide structure (Figure 4.2) for recognition and transportation by the intestinal oligopeptide transporter [Kramer *et al.*, 1995], through conclusions drawn from a series of studies investigating cephalexin uptake into rabbit small intestinal BBMV and photoaffinity labelling of the oligopeptide transporter protein.

FIGURE 4.2 *Hypothetical peptide structure for recognition and transportation by the intestinal oligopeptide transporter (taken from Kramer et al., 1995)*

R_2 of the proposed structure is recommended to be a small electrically neutral substituent (*e.g.* H, CH_3 or vinyl). X should be capable of accepting protons in a proton donor-acceptor relationship with the histidyl of the transporter, and is recommended to have a pKa between 6.5 and 7.5 (*e.g.* NH_2, imdazolyl, or a thiol). Details for R_1 and R_3 are not given, although it is suggested that R_3 should not be a negatively charged group, with n being 0 or 1.

4.2 MATERIALS AND METHODS

4.2.1 MATERIALS

Details of the suppliers of all materials used throughout this chapter have been described previously (section 2.1-2.1.3).

4.2.2 METHODS

4.2.2.1 MEDIA

Details of growth media (M1), and incubation media (M4) used throughout this chapter have been described previously (section 2.2.1.1).

4.2.2.2 CELL CULTURE

Cell line-1 (section 2.1.1) was used between passage number 25 to 38. The cells were seeded onto 24-well plates (2 cm^2) at a density of 1.6 x 10^5 cells $well^{-1}$, and grown in an atmosphere of 5 % CO_2 (95 % air) and 90 % relative humidity, at 37°C, as previously described (section 2.2.1.3). M1 was renewed every 48 h and the monolayers were used after 7 d growth, unless otherwise stated.

4.2.2.3 EXPERIMENTAL CONDITIONS FOR UPTAKE STUDIES

The following procedure and conditions were used for all studies unless otherwise stated: All solutions were preincubated at 37°C prior to experimentation, with the exception of PBS-azide which was kept at 4°C. The maintenance media was aspirated and the monolayers were washed with M4 at pH 6 (1 x 500 µl x 5 min) at 37°C. The incubation solution consisted of M4 with 2 % DMSO at pH 6, containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro, and the required amount of competitor (0.025-10 mM). Monolayers were incubated (1 x 250 µl x 3 min), at 37°C. Apical solutions were then collected, and the plates transferred to a cold table (4°C) and washed with ice-cold PBS-azide solution (2 x 500 µl x 5 min). The monolayers were then harvested by solubilising with 1 ml of 1 % v/v TX-100 in double distilled water. Apical solutions, washings and solubilised cells were collected made up to 1 ml with double distilled water, added to 15 ml Ecolite$^+$, and counted for ^{3}H content by LSC. SQ-29852 was included as a competitor for each experiment and tested at a concentration of 1 mM. If the inhibition for SQ-29852 was outside the range (60.51 ± 6.73 %) previously reported (section 3.3.2), the experiment was repeated. Results were corrected for 15 % non-specific uptake, and expressed % inhibition from the control value (Gly-[^{3}H]L-Pro uptake in the absence of competitor) and IC_{50} (mM) (Chapter 3, Equation 3.4). Data are mean (of at least three monolayers) ± S.D. Competitors which produced inhibition values lower than 15 % or higher than 85 % were retested at a higher or lower concentration respectively, in order to achieve values within this range, where possible. Some of the results are incomplete and ideally require repeating. However, solubility problems restricted the testing of some analogues at the correct concentration, and availability of compounds was also a problem. Where compounds were tested at more than one concentration, all results are shown. x and y correspond to inhibition values (%) which are too large (≥ 100.00) or too small (≤ 0.00), respectively, to be converted into a relevant IC_{50} values. Ideally, these compounds were retested at a lower or higher concentration where possible.

4.2.3 STRUCTURAL REQUIREMENTS FOR THE DTS

4.2.3.1 EVALUATION OF BINDING DOMAIN A

The optimum structure for binding in domain A was evaluated by using a series of SQ-29852 analogues were the phenylbutyl group had been substituted, with all other domains being kept constant where possible (Table 4.1).

4.2.3.2 EVALUATION OF BINDING DOMAIN C

Structural requirements for domain C were investigated using series of Gly-L-Pro analogues where the amino group was substituted (Table 4.2), and a series of SQ-29852 analogues with substitutions for the butylamine group (Table 4.3).

4.2.3.3 EVALUATION OF BINDING DOMAIN D

Structural requirements for binding domain D were investigated using two series of SQ-29852 analogues. The first series comprised of compounds with substitutions made at position 4 of the proline ring (Table 4.4), and the second where the proline ring itself was substituted (Table 4.5).

4.2.3.4 THE INTERACTION OF VARIOUS GLY-L-PRO ANALOGUES WITH THE DTS

A series of compounds which are variations on the Gly-L-Pro structure were tested to assess their interaction with the DTS (Table 4.6).

4.2.3.5 MISCELLANEOUS COMPETITORS

Three SQ-29852 analogues which did not classify for a specific group, yet are directly comparable to previous SQ-29852 analogues, were tested. The tripeptide glycyl-L-prolyl-L-arginine (Gly-L-Pro-L-Arg) was also tested for affinity with the DTS (Table 4.7).

4.3 RESULTS AND DISCUSSION

Fifty four compounds which comprised Gly-L-Pro analogues and SQ-29852 analogues were selected from a Bristol-Myers Squibb library consisting of over 5000 compounds. The analogues have been divided into groups based on the domain (Figure 4.1) in which the structural variations occur. Compounds with structural variations in the B and F domain were unobtainable and therefore no conclusions within these areas can be reached. The information for domain E is also limited due to the desired analogues being unavailable.

4.3.1 DOMAIN A

The structural requirements of binding region A (Figure 4.1) were explored by replacing the phenylbutyl group with various other structures (Table 4.1). SQ-29943, which

has a hexylamine group (pKa of amine is approximately 10.8, based on literature value for the side-chain of Lys) substituted, giving domain A a positive charge, and making the molecule a zwitterion, shows a reduction in affinity for the DTS, as shown by the large increase in the IC_{50} value from 0.68 ± 0.17 mM to 43.94 ± 8.24 mM, compared to that of SQ-29852. This suggests that a large hydrophobic group is preferred in this region. SQ-30640, which is SQ-29943 with the butylamine group in domain C being replaced with a methyl group, shows no affinity for the transporter at the concentration tested (1 mM), even though SQ-30640 has the same overall net charge as SQ-29852. Retesting is required to assess whether it shows interaction at a higher concentration. SQ-30922, which has a benzoyl group attached to the phenylbutyl chain and, therefore has H^+-acceptor properties, again shows less inhibition, with an IC_{50} value of 3.06 ± 0.51 mM, although not reduced to the extent shown by of SQ-29943. SQ-30288, which is similar to SQ-30922, but with the addition of an amide group making it a H^+-acceptor and donor, showed reduced recognition with the transporter, producing an IC_{50} value of 13.91 ± 2.16 mM. Region A preferred a hydrophobic group for optimal binding, addition of a positive charge or H^+-acceptors or donors resulted in substantially reduced recognition, however the DTS seems more tolerant of H^+- acceptors than donors, as shown by the difference in IC_{50} values for compounds SQ-30922 (3.06 ± 0.51 mM) and SQ-30288 (13.91 ± 2.16 mM). Interestingly, bulky hydrophobic groups have been shown to enhance interaction between ACE-inhibitors and ACE [Pascard *et al.*, 1991].

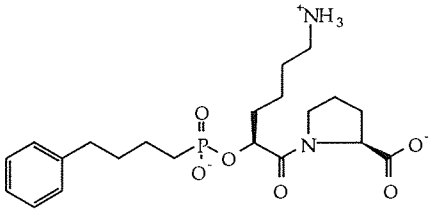
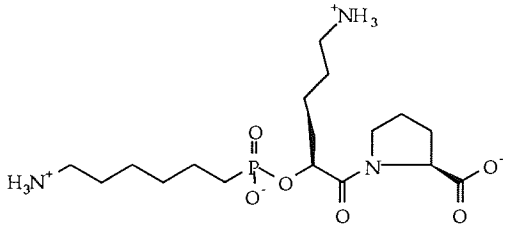
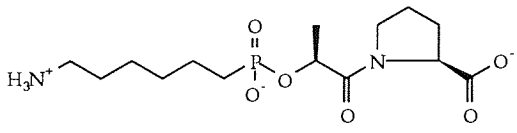
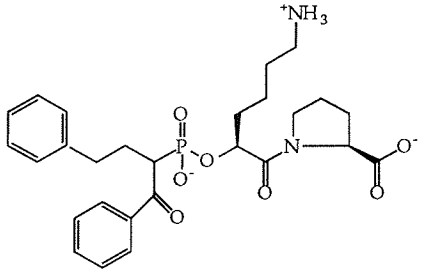
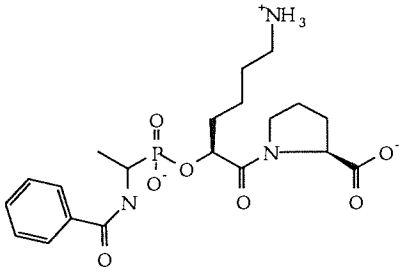
4.3.2 DOMAIN C

The structural requirements of the C domain were investigated by using two sets of competitors, Gly-L-Pro (Table 4.2) and SQ-29852 (Table 4.3) analogues. The effect of weak bases in the C domain was explored by Gly-L-Pro analogues (Table 4.2). SQ-13870 has the amino chain extended by the addition of a methylene group, which resulted in reduced inhibition when compared to Gly-L-Pro, with IC_{50} values of 0.59 ± 0.02 mM and 0.17 ± 0.02 mM respectively. Sar-L-Pro (Chapter 3, Table 3.4), which is essentially the same structure as SQ-13870, with a secondary rather than a primary amino group, produced an IC_{50} value of 3.51 ± 0.97 mM compared to 0.59 ± 0.02 mM for SQ-13870. This illustrates that the DTS has a preference for primary amino groups at the *N*-terminus. When the amino group is extended further by the addition of a further methylene group to the chain, as in SQ-13880, again affinity is reduced (IC_{50} = 2.94 ± 1.17 mM). SQ-26483, with the imidazole moiety, which is a weaker base compared to the amino group (5-membered ring containing two nitrogens, pKa of approximately 6.04, based on literature value for His side chain), showed a reduction in affinity, producing an IC_{50} value of 8.11 ± 3.42 mM. This is probably due to the fact that this group is bulky and conformationally restricted. However, although for SQ-13880 and SQ-26483, the difference in IC_{50} values (2.94 ± 1.17 mM, 8.11 ± 3.42 mM) appear to be large compared to the control value, the results are not quite significantly different from control, at the 95 % confidence limits; this is due to the

large standard deviations. Note that SQ-26483 produced only 11.99 ± 4.09 % inhibition, and therefore, in order to achieve an accurate IC_{50} value, it needs retesting at a higher concentration. However, the present data give an idea of the range in which the affinity falls. SQ-25269, has a methylene group joining the imidazole moiety to the core structure, resulting in a total loss of affinity for the transporter. SQ-26220 has the same structure as SQ-25269 with the addition of another methylene group joining the imidazole and, again, shows no affinity for the transporter.

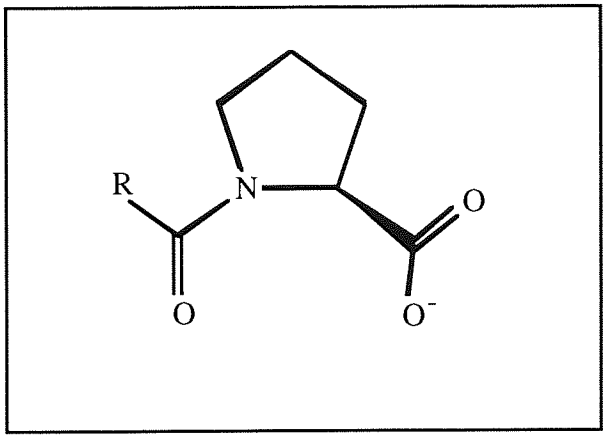
N-Boc-L-Pro, which has the amino group substituted with a tetrabutyl ester (neutral side chain) and SQ-25135 which has the amino group substituted with a primary alcohol, show no affinity for the transporter at 1 mM. Both compounds need retesting at a higher concentration (10 mM) to assess whether they show any affinity for the DTS. *N*-acetyl-L-Pro which has a methyl group in place of the amino group, still displays affinity for the transporter although it is greatly reduced. Replacing the amine group with an acid function, as in SQ-13964, SQ14568, SQ-13926, *N*-Succinyl-L-proline and SQ-25000, produced a total or considerable reduction in affinity. In conclusion, extending the amino group or substituting it with either weak bases, neutral or acidic functions produced a significant reduction in affinity, with all the analogues tested displaying less affinity for the DTS than the control compound, Gly-L-Pro.

TABLE 4.1 *Evaluation of binding domain A*

Compound	Conc (mM)	Structure	% Inhibition Mean [S.D.]	IC_{50} mM Mean [S.D.]
SQ-29852	1		60.51 [6.73]	0.68 [0.17]
SQ-29943	10		18.84 [3.01]	43.94 [8.24][c]
SQ-30640	1		-1.15 [4.68]	y
SQ-30922	1		24.86 [2.95]	3.06 [0.51][a]
SQ-30288	10		42.03 [3.62]	13.91[2.16][b]

Monolayers were washed (1 x 500 µl x 5 min) with M4. Then incubated with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6 in the presence of competitor (for concentration see table). Data are corrected for 15 % non-specific uptake and presented as mean % inhibition and IC_{50} ± S.D. for three monolayers, with [a], [b], and [c] denoting significant difference at $p< 0.0001$, $p< 0.01$ and $p< 0.05$ respectively, in IC_{50} values compared to the control (uptake in the presence of 1 mM SQ-29852). y corresponds to a % inhibition value which is too small (% inhibition ≤ 0.00) to be converted into a relevant IC_{50} value.

TABLE 4.2 *Evaluation of binding domain C, using Gly-L-Pro analogues*



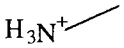
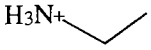
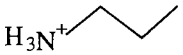
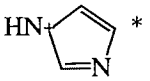
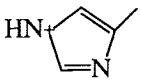
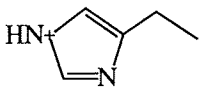
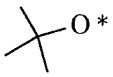
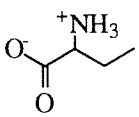
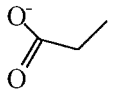
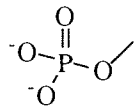
Compound	R Group Substituent	% Inhibition Mean [S.D.]	IC_{50} mM Mean [S.D.]
Gly-L-Pro		85.18 [1.31]	0.17 [0.02]
SQ-13870		62.89 [0.58]	0.59 [0.02][a]
SQ-13880		26.88 [7.69]	2.94 [1.17][c]
SQ-26483		11.99 [4.09]	8.11 [3.42][c]
SQ-25269		-29.88 [7.25]	y
SQ-26220		-8.86 [1.67]	y
N-Boc-L-Pro		-4.01 [6.11]	y
N-Acetyl-L-Pro	CH_3	A-10.89 [4.79] B-36.27 [6.65]	92.45 [36.35][b] 91.41 [28.97][b]
SQ-25135		-8.05 [12.09]	y
SQ-13964		12.59 [10.39]	y
SQ-14568		-3.77 [3.76]	y

TABLE 4.2 *Evaluation of binding domain C, using Gly-L-Pro analogues, continued*

SQ-13926		14.14 [3.62]	6.36 [1.68]^{b}
N-Succinyl-L-proline		21.81 [7.06]	3.88 [1.35]^{b}
SQ-25000		-2.34 [1.55]	y

* Attachment to the structure through this atom.

Monolayers were washed (1 x 500 µl x 5 min) with M4. They were incubated with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6 in the presence of 1 mM competitor (except *N*-Acetyl-L-Pro, which was tested at 10 mM {A} and 50 mM {B}). Data are corrected for 15 % non-specific uptake and presented as mean % inhibition ± S.D. and IC_{50} ± S.D. for three monolayers, with ^{a} and ^{b} denoting significant difference at $p < 0.0001$ and $p < 0.05$ respectively, in IC_{50} values compared to the control value (uptake in the presence of 1 mM Gly-L-Pro). ^{c} denotes not quite a significant difference at the confidence limits tested (95 %). y correspond to a % inhibition value which is too small (% inhibition ≤ 0.00) to be converted into a relevant IC_{50} value.

SQ-29852 analogues (Table 4.3) appear to be more tolerant of changes in the C domain when compared to the smaller Gly-L-Pro analogues, which suggests that other interactions along the length of the molecule are important. SQ-30711, has the amino group extended by a single methylene group, which results in the total loss of inhibition at the concentration tested (1 mM). This indicates that the binding site in this region may not be large enough to accommodate this structure. When the amino group is removed, as in SQ-29907, the affinity is again reduced compared to SQ-29852 (0.68 ± 0.17 mM to 1.24 ± 0.27 mM). This suggests that a positive charge in this region is preferred. Further replacement of the amine with a guanidino group SQ-30403, which is a stronger base than the primary amino group (pKa of approximately 12.48, based on a literature value for the side-chain of Arg) sees a reduction in binding when compared to SQ-29852. However, extending the chain from a butyl to pentyl (SQ-30472) increases affinity by a factor of two compared with SQ-30403. SQ-30711 has a 5-carbon chain in the C domain and shows no affinity for the transporter, however, when the amine was replaced with a guanidino group (SQ-30403), affinity for the DTS is seen, IC_{50} of 3.14 ± 1.01 mM. This suggests that the chain length is not an important parameter, but that the space the structure occupies and the surface area available for interaction with the transporter binding site is a key factor.

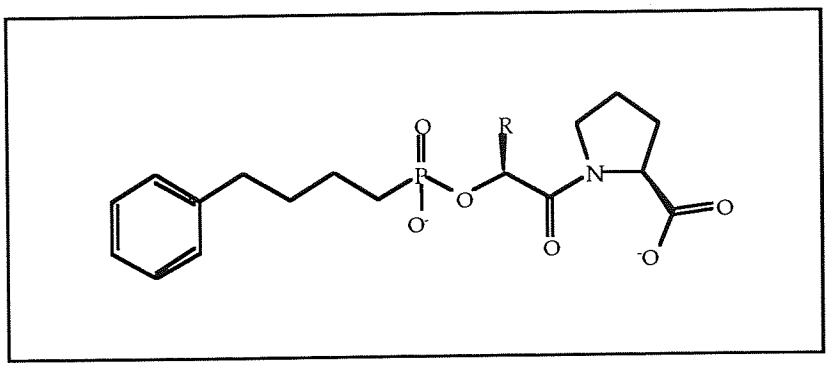
4.3.3 DOMAIN D

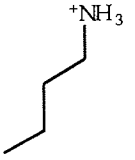
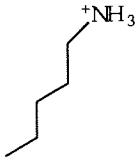
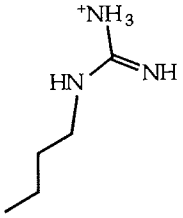
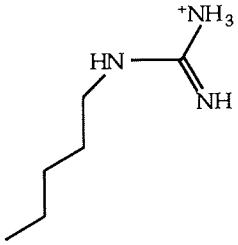
Previously, all analogues tested had caused a reduction in affinity for the DTS. However, substitutions in the D domain (Table 4.4) produced an increase in recognition when compared to the original compound, SQ-29852. All of the analogues tested which had functional groups substituted at position 4 of the proline ring had better affinity for the transporter than the control compound, with some analogues also having better affinity than the unlabelled probe, Gly-L-Pro. SQ-30427, in which a hydroxy group replaces the hydrogen, appeared to show a slight increase in affinity for the DTS, however the IC_{50} value was not quite significantly different from that of the control, at the 95 % confidence limits tested. When a lipophilic, aliphatic group such as cyclohexyl is added to the 4-position on the proline ring, as in SQ-30071, there is at least a 10-fold increase in recognition compared to the control compound. SQ-29841, which is the enantiomer of SQ-30071, had greater affinity (IC_{50} = 0.09 ± 0.04 mM) than SQ-29852 (IC_{50} = 0.68 ± 0.12 mM), but less than that of SQ-30071 (IC_{50} = 0.05 ± 0.02 mM), indicating a stereochemical preference for the functional group at this position. SQ-31423, having a methyl group at position 4 showed better affinity (IC_{50} = 0.23 ± 0.03 mM) than the control. The addition of an extended methyl group with a benzyl substitution (SQ-30361) again had better affinity (IC_{50} = 0.13 ± 0.03 mM) than SQ-29852. SQ-30361 displayed a lower affinity for the transporter compared to SQ-30071, IC_{50} values of 0.13 ± 0.03 mM and 0.05 ± 0.02 mM respectively, suggesting that the methylene group added has a negative effect. The addition of a thiomethyl group at position 4 (SQ-30381) produced greater than a 10-fold increase in affinity, when compared to SQ-29852. SQ-30366, the enantiomer of SQ-30381, had a

reduction in recognition, which is in agreement with previous results demonstrating a stereochemical preference at position 4. SQ-30265, has a similar structure to SQ-30361, with a thiol group in place of the methylene. The IC_{50} values for SQ-30265 and SQ-30361 are identical, indicating that substituting the carbon for a sulphur has no effect on affinity. SQ-28143, which has a substituted 2, 5 dithiocyclopentyl group, produced an IC_{50} which was not significantly different from that of the control while SQ-30851, which has a dithiomethyl group substituted at position 4, was found to have the greatest affinity for the DTS from all the compounds tested ($IC_{50} = 0.01 \pm 0.00$). Dithiol groups at position 4, have been shown to be compatible with the DTS, and improve recognition; however, the 2,5 dithiocyclopentyl group did not follow this trend. The ring structure at position 4 in SQ-28143 is more conformationally constrained, which may explain the absence of increased affinity.

The majority of substitutions made to or for the proline ring (Table 4.5) also show a marked improvement in recognition with the DTS. A methyl group added to position 3 of the proline ring (SQ-31053) was the only compound within this series that resulted in a decrease in affinity. When a methyl group is added to position 5 (SQ-30859), affinity is not significantly improved but it is not reduced as in SQ-31053. However, when methyl groups are added to position 4 and 5, as in SQ-31614, affinity is increased. SQ-31614 is directly comparable to SQ-31423 (Table 4.4). A methyl group at position 4 (SQ-31423) shows an increase in affinity, a methyl group at position 5 (SQ-30859) shows no increase but methyl groups at 4 and 5 (compound SQ-31614) produce an even greater inhibition, therefore showing a cumulative effect. Both 4, 5-cyclopentyl fusion (SQ-31479) and a 4, 5-cyclohexyl fusion (SQ-30231) with the proline ring, show an identical increase in affinity when compared to SQ-29852. SQ-30191, which has an *N*-cyclohexyl glycine group substituted for the proline, and SQ-30229 which has an *N*-benzocyclopentyl in place of the proline both showed an increase in recognition, IC_{50} values of 0.33 ± 0.04 mM and 0.24 ± 0.02 mM respectively. SQ-30191 and SQ-30229 both have lower affinities for the DTS than SQ-31479 ($IC_{50} = 0.06 \pm 0.003$ mM) and SQ-30231 ($IC_{50} = 0.06 \pm 0.01$ mM), which can be explained by the fact that they have free rotation about the nitrogen atom giving rise to more conformations. These results illustrate that a hydrophobic constrained group is preferred at this position of the molecule, as demonstrated by SQ-31479 and SQ-30231. However, this is not in agreement with observations for SQ-30851, which had considerably greater affinity ($IC_{50} = 0.01 \pm 0.00$ mM) than a constrained analogue, SQ-28143 ($IC_{50} = 0.73 \pm 0.09$ mM).

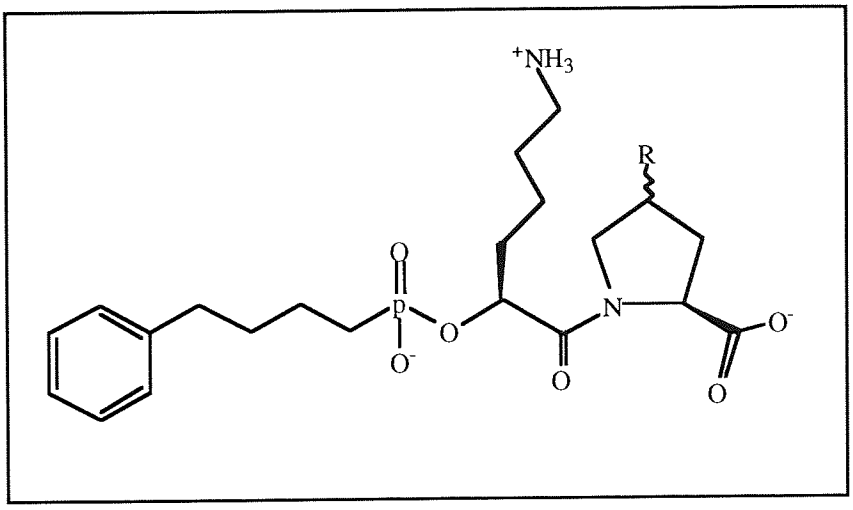
TABLE 4.3 *Evaluation of binding domain C, using SQ-29852 analogues*



Compound	Conc (mM)	R Group Substituent	% Inhibition Mean [S.D.]	IC_{50} mM Mean [S.D.]
SQ-29852	1		60.51 [6.73]	0.68 [0.17]
SQ-30711	1		0.63 [5.37]	y
SQ-29907	10		88.97 [2.14]	1.24 [0.27][b]
SQ-30403	1		25.08 [5.79]	3.14 [1.01][c]
SQ-30472	1		38.23 [2.39]	1.62 [0.16][a]

Monolayers were washed (1 x 500 µl x 5 min) with M4, then incubated with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6 in the presence of competitor (for concentration see table). Data are corrected for 15 % non-specific uptake and presented as mean % inhibition ± S.D. and IC_{50} ± S.D. for three monolayers, with, [a] and [b] denoting significant difference at $p < 0.005$ and $p < 0.05$ respectively, in IC_{50} values compared to the control value (uptake in the presence of 1 mM SQ-29852). [c] denotes not quite a significant difference from the control at the confidence limits tested (95 %). y corresponds to a % inhibition value which is too small (% inhibition ≤ 0.00) to be converted into a relevant IC_{50} value.

TABLE 4.4 *Evaluation of binding domain D (1), using analogues of SQ-29852*



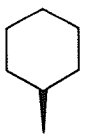
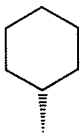
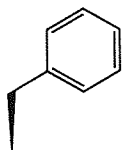
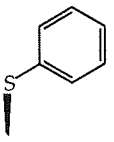
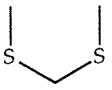
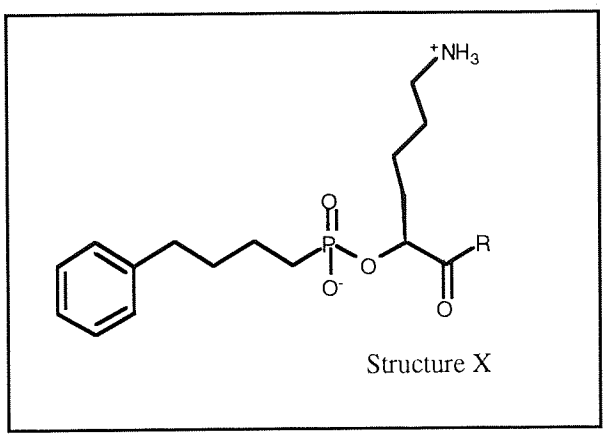
Compound	Conc (mM)	R Group Substituent	% Inhibition Mean [S.D.]	IC50 mM Mean [S.D.]
SQ-30427	1		67.69 [5.41]	0.48 [0.12][c]
SQ-30071	1		102.38 [0.51]	x
	0.5		99.13 [0.79]	x
	0.1		66.29 [7.67]	0.05 [0.02][b]
SQ-29841	1		91.68 [3.54]	0.09 [0.04][a]
SQ-31423	1		81.05 [1.663]	0.23 [0.03][b]
SQ-30361	1		88.24 [2.10]	0.13 [0.03][b]
SQ-30381	1		102.52 [0.19]	x
	0.1		66.44 [1.85]	0.05 [0.00][b]
SQ-30366	1		78.52 [1.85]	0.27 [0.03][b]

TABLE 4.4 *Evaluation of binding domain D (1), using analogues of SQ-29852, continued*

SQ-30265	1		88.66 [1.67]	0.13 [0.02][b]
SQ-28143	1		58.05 [3.02]	0.73 [0.09][c]
SQ-30851	1		106.86 [2.18]	x
	0.1		95.45 [0.70]	0.005[0.001][b]
	0.025		67.71 [1.69]	0.01 [0.00][b]

Monolayers were washed (1 x 500 µl x 5 min) with M4. They were incubated with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6 in the presence of competitor (for concentration see table). Data are corrected for 15 % non-specific uptake and presented as mean % inhibition ± S.D. and IC_{50} ± S.D. for three monolayers, with [a] and [b] denoting significant difference at $p< 0.005$ and $p< 0.05$ respectively in IC_{50} values compared to the control value (uptake in the presence of 1 mM SQ-29852). [c] denotes not quite a significant difference at the confidence limits (95 %) tested. x corresponds to an inhibition value which is too large (% inhibition ≥ 100.00) to be converted into a relevant IC_{50} value.

TABLE 4.5 *Evaluation of binding domain D (2), using analogues of SQ-29852*



Structure X

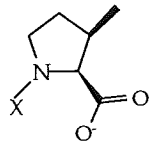
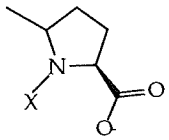
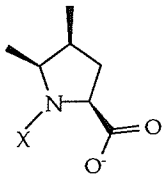
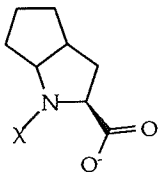
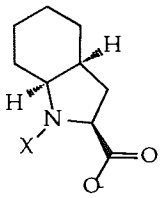
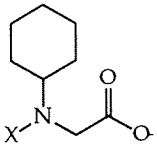
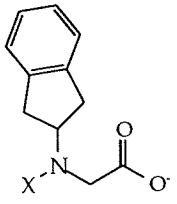
Compound	Conc (mM)	R Group Substituent	% Inhibition Mean [S.D.]	IC_{50} mM Mean [S.D.]
SQ-31053	1		47.99 [2.59]	1.09 [0.11]
SQ-30859	1		67.36 [2.24]	0.49 [0.05][b]
SQ-31614	1		86.53 [0.21]	0.16 [0.003][a]
SQ-31479	1 0.1		99.48 [0.82] 62.37 [1.31]	x 0.06 [0.003][a]
SQ-30231	1 0.1		98.47 [2.67] 61.62 [3.78]	x 0.06 [0.01][a]
SQ-30191	1		75.43 [2.31]	0.33 [0.04][a]

TABLE 4.5 *Evaluation of binding domain D (2), using analogues of SQ-29852, continued*

SQ-30229	0.5		67.51 [1.42]	0.24 [0.02] [a]

Attachment to the core structure is through the nitrogen atom of all functional groups.

Monolayers were washed (1 x 500 µl x 5 min) with M4. They were incubated with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6 in the presence of competitor (for concentration see table). Data are corrected for 15 % non-specific uptake and presented as mean % inhibition ± S.D. and IC_{50} ± S.D. for three monolayers, with [a] denoting significant difference at $p < 0.05$ and [b] being not quite a significant difference at the confidence limits (95 %) tested, in IC_{50} values compared to the control value (uptake in the presence of 1 mM SQ-29852). x corresponds to an inhibition value which is too large (% inhibition ≥ 100.00) to be converted into a relevant IC_{50} value.

4.3.4 THE EFFECT OF VARIOUS GLY-L-PRO ANALOGUES

Again, all the analogues tested produced a reduction in affinity for the DTS compared to that seen with Gly-L-Pro (Table 4.6). By substituting an hydroxy group at position 4 of the proline ring (Gly-L-Pro-OH), affinity for the transporter was abolished at the concentration tested. Addition of an hydroxy group at position 4 of SQ-29852 (SQ-30427) had no effect on the overall affinity of the molecule (Table 4.4), indicating that, with smaller molecules such as Gly-L-Pro, substitutions to the proline ring have a greater impact. However, this may also be explained by the fact that interactions with the DTS along the length of the SQ-29852 molecule override the effect of the hydroxy substitution in the proline ring. Inhibition values for SQ-26837, *N*-acetyl-L-prolinamide, *N*-acetyl-L-hydroxyprolinamide, *N*-Boc-L-Pro-t-butylester and the proline dimer, all fall below 15 % and therefore, in order to assess affinity they need retesting at a higher concentration. However, these preliminary results all illustrate that these molecules, which lack the *N*-terminal amino group show considerably less affinity for the DTS compared to Gly-L-Pro, which agrees with previous observations [Matthews, 1987]. *N*-Boc-L-prolinamide, with *N*-Boc in place of the amine, retains affinity for the DTS, although it is reduced from the control compound (Gly-L-Pro). 1-Methyl-2-pyrolidineethanol, which lacks both the amine and carboxy functions, and is not a peptide, stills show some affinity, although reduced, for the transporter, giving an IC_{50} value of 1.41 ± 0.20 mM. Modification of the carboxylic acid (Domain E) with a tetrabutyl ester, (BMY-46679) results in a > 50 % reduction in affinity compared to Gly-L-Pro, in agreement with a previous study using L-Val-L-Val [Hidalgo *et al.*, 1995].

4.3.5 MISCELLANEOUS COMPETITORS

Four compounds (Table 4.7) were tested which did not fall into any specific grouping yet bore similarities with certain structures. SQ-31065, is SQ-29852 with the *C*-terminal carboxy group substituted with a benzoyl ester. SQ-31065 has a significantly greater affinity for the transporter (IC_{50} value of 0.05 ± 0.01 mM), when compared directly to SQ-29852. Thus illustrating that a negative charge and/or a free carboxy group in this region (Domain E) are not required for recognition. This is contradictory to previous reports stating a free carboxy group is required for optimal interaction [Hidalgo *et al.*, 1995]. Addition of an Arg (Gly-L-Pro-L-Arg) resulted in a loss of affinity compared to Gly-L-Pro, indicating that an anionic interaction is preferred in domain E, for this type of compound. SQ-30927, is SQ-30403 (Table 4.3) with an hydroxy group substituted at position 4 in the proline ring. The IC_{50} values, 2.60 ± 0.72 mM and 3.14 ± 1.10 mM for SQ-30927 and SQ-30403 respectively, are not significantly different from on another at the confidence limits tested (95 %). These results are in agreement with those previously seen for SQ-29852 and SQ-30427, which illustrated that addition of an hydroxy group to the proline ring in SQ-29852 analogues has no significant effect on interaction with the DTS. SQ-29817, is SQ-29841 (Table 4.4), with the butylamine group in domain C being replaced with a methyl

group. There is a significant reduction in affinity for the DTS (IC_{50} values of 0.09 ± 0.04 mM for SQ-29841 compared to 0.87 ± 0.07 mM for SQ-29817) with the removal of the butylamine group (positive charge), indicating that the presence of a positive charge is essential for optimum interaction.

4.3.6 HYPOTHETICAL PEPTIDE STRUCTURE

A hypothetical peptide structure for recognition and transportation by the DTS, has been proposed (Figure 4.2) [Kramer *et al.*, 1995]. It suggests that domain D (R_2 in their nomenclature), should be a small, electrically neutral substituent such as H, CH_3 or vinyl. However, we have shown that large lipophilic, aliphatic groups such as cyclohexyl (SQ-30071) and *N*-cyclohexyl glycine (SQ-30229) are well tolerated, with SQ-30071 (IC_{50} = 0.05 ± 0.02 mM) and SQ-30229 (IC_{50} = 0.24 ± 0.02 mM), illustrating greater affinity for the transporter than the parent compound, SQ-29852 (IC_{50} = 0.68 ± 0.17 mM). Kramer *et al.*, suggest domain C (R_3 in their nomenclature), must not be a negatively charged group which is in agreement with the present study, where SQ-13964, SQ-14568, SQ-13926, *N*-succinyl-L-proline and SQ-25000 all showed poor affinity. However, we propose that a positively charged group at physiological pH, such as butylamine, is the most suitable group for this region as shown by the data recorded in Table 4.3. The length of this group also seems important in the extent of recognition with the DTS. Kramer *et al.*, did not speculate a possible structure for R_1 , which corresponds to the A domain in our model, but we found that a neutral group, such as phenyl is preferred over positively charged moieties (*e.g.* amino group) and that H^+- acceptors and -donors are not favoured.

TABLE 4.6 *The effect of various Gly-L-Pro analogues*

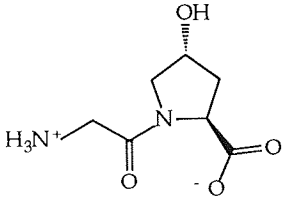
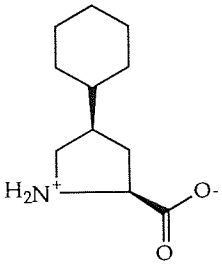
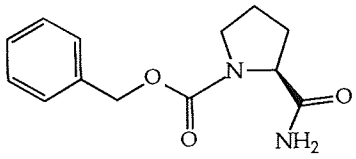
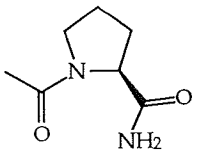
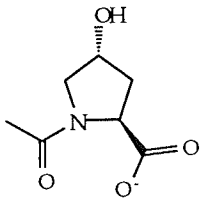
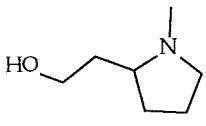
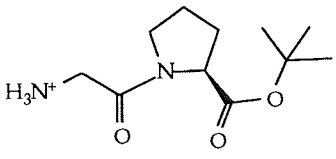
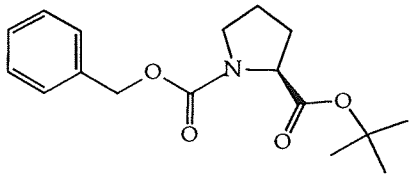
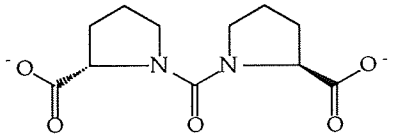
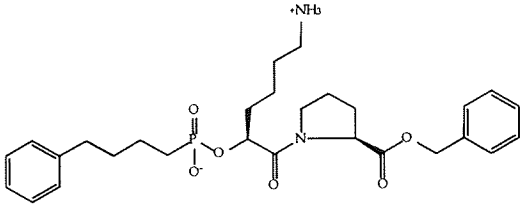
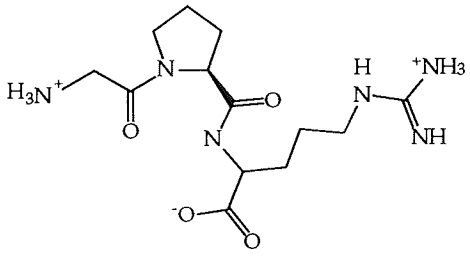
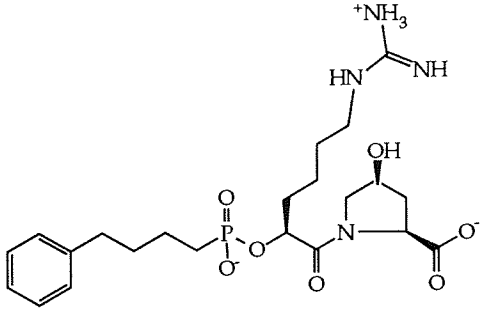
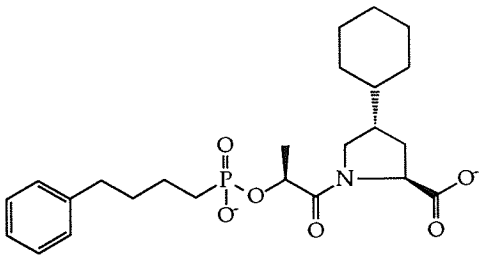
Compound	Conc (mM)	Structure	% Inhibition Mean [S.D.]	IC_{50} mM Mean [S.D.]
Gly-L-Pro-OH	1		-25.15 [24.42]	y
SQ-26837	1		9.20 [3.72]	11.31[5.46][d]
N-Boc-L-prolinamide	1		48.56 [3.65]	1.06 [0.16][c]
N-Acetyl-L-prolinamide	1		4.56 [1.77]	24.18[12.55][d]
N-Acetyl-L-hydroxyproline	1		13.55 [3.53]	6.68 [1.75][c]
1-methyl-2-pyrolidine-ethanol	1		41.74 [3.37]	1.41 [0.20][b]
BMY-46679	1		32.42 [1.63]	2.09 [0.16][a]

TABLE 4.6 *The effect of various Gly-L-Pro analogues, continued*

N-Boc-L-Pro-t-butylester	0.5		15.54 [2.05]	2.75 [0.40][b]
Proline dimer	1		14.30 [5.93]	7.22 [4.44][d]

Monolayers were washed (1 x 500 µl x 5 min) with M4. They were incubated with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6 in the presence of competitor (for concentration see table). Data are corrected for 15 % non-specific uptake and presented as mean % inhibition ± S.D. and IC_{50} ± S.D. for three monolayers, with [a], [b] and [c] denoting significant difference at $p < 0.005$, $p < 0.01$ and $p < 0.05$ respectively, in IC_{50} values compared to the control value (uptake in the presence of 1 mM Gly-L-Pro). [d] denotes not quite a significant difference at the confidence limits (95 %) tested. y corresponds to a % inhibition value which is too small (% inhibition ≤ 0.00) to be converted into a relevant IC_{50} value.

TABLE 4.7 *Miscellaneous table of competitors*

Compound	Conc (mM)	Structure	% Inhibition Mean [S.D.]	IC_{50} mM Mean [S.D.]
SQ-31065	0.5		90.41[1.90][a]	0.05 [0.01]
Gly-L-Pro-L-Arg	10		86.15[0.56][b]	1.61 [0.08]
SQ-30927	1		28.54[5.99][c]	2.60 [0.72]
SQ-29817	1		53.41[2.06][a]	0.87 [0.07]

Monolayers were washed (1 x 500 µl x 5 min) with M4. They were incubated with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6 in the presence of competitor (for concentration see table). Data are corrected for 15 % non-specific uptake and presented as mean % inhibition ± S.D. and IC_{50} ± S.D. for three monolayers, with [a], [b] and [c] denoting significant difference at $p < 0.0001$, $p \leq 0.001$ and $p < 0.005$ respectively, in % inhibition values from the control value (uptake in the absence of competitor).

4.4 CONCLUSION

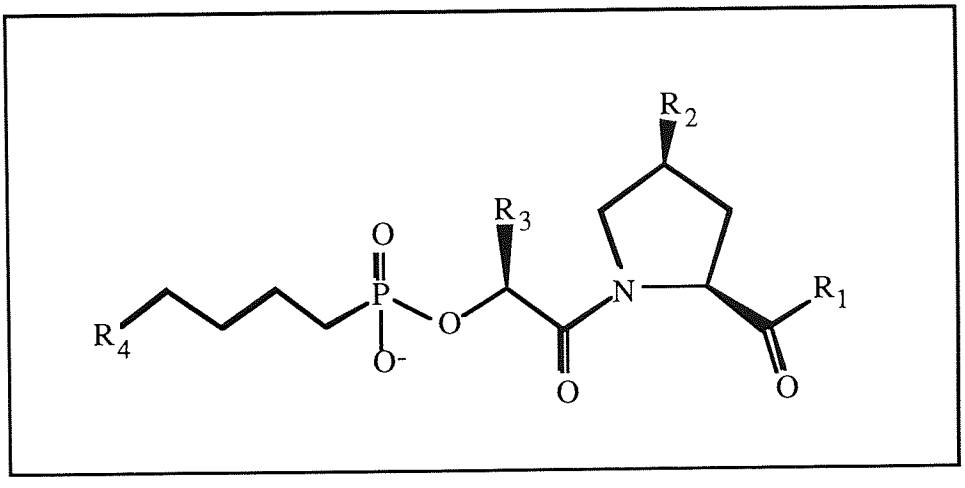
Domain A showed a preference for a hydrophobic group for optimum interaction with the DTS. A positive charge, along with H^+-acceptors and donors were not well tolerated, resulting in a significant reduction in affinity for the DTS.

All modification and substitutions made in domain C of the Gly-L-Pro structure, resulted in a reduced affinity for the transporter. Extending the amine group or substituting with weak bases, neutral or acidic functions decreased affinity. The weaker the base substituted at C, the lower the affinity. SQ-29852 analogues were more tolerant of modifications in domain C. A preference for a positive charge (amine group) in this region was demonstrated. The space that a functional group occupies appears to be more important for interaction than the length of the carbon chain in this area.

All analogues with substitutions at position 4 of the proline ring, showed better affinity for the DTS compared to the control compound, SQ-29852, and in some cases the unlabelled probe, Gly-L-Pro. A stereochemical preference for the L-configuration of the functional group was demonstrated. Lipophilic aliphatic groups, such as cyclohexyl were preferred, showing a 10-fold improvement in recognition compared to SQ-29852. The analogue SQ-30851, with a dithiomethyl group at position 4 of the proline ring had the greatest affinity for the DTS, of all compounds tested. The majority of analogues with substitutions of the proline ring resulted in an increase in recognition. A preference for hydrophobic constrained groups, as in SQ-31479 and SQ-30231, were demonstrated.

Modification of the carboxy group in domain E as seen in BMY-46679 (Gly-L-Pro analogue) resulted in a decrease in affinity. However, when a similar modification was made to the SQ-29852 analogue, SQ-31065, an increase in affinity is observed. Further analogues with specific structural variations in domain E are required to clarify the functional groups required for maximum interaction in this region.

FIGURE 4.3 *Proposed hypothetical peptide-like structure for optimum interaction with the DTS (1)*



Information gained form the present study, has enabled a hypothetical peptide structure based on the SQ-29852 structure to be suggested (Figure 4.3). In conclusion, R_1 does not have to be a negatively charged group, as illustrated by SQ-31065, and a hydrophobic group, such as phenyl, may increase interaction, although further information is needed in this area. R_2 is suggested to be a hydrophobic group (as shown by the data in Table 4.4), while the most appropriate functional group for R_3 appears to possess a positive charge (*e.g.* NH_3^+). However, chain length and the space that the group occupies may also be factors that need to be investigated. R_4 is suggested to be a neutral hydrophobic function.

CHAPTER 5

STEREOCHEMICAL PREFERENCE OF THE DTS AND THE EFFECT OF CHARGE IN THE C DOMAIN ON INTERACTION WITH THE DTS

ABSTRACT

Dipeptides consisting of L-enantiomers were found to be the preferred enantiomer for the DTS. Dipeptides consisting exclusively of D-enantiomers displayed reduced affinity for the DTS compared to the L, L version of the dipeptide. However, in some instances dipeptides comprised of mixed isomers had the same affinity for the DTS as the D, D isomers. D-Ala-D-Ala was the only dipeptide tested that failed to inhibit uptake, which is surprising considering that the cephalosporins, bearing a resemblance to this structure, are often used as probes for the intestinal peptide transporter. The position of the D-amino acid in a mixed isomer dipeptides (L, D or D, L) had differing results depending on dipeptide composition. A D-amino acid at the *C*-terminal position in L-Lys-D-Pro produced the greatest reduction in affinity, with an IC_{50} value of 29.94 ± 3.18 mM. Whereas, for Ala-Pro and SQ-29852 isomers inclusion of a D-amino acid (or R) at the *N*-terminal had a greater impact. SQ-32009, which is the S-R version of SQ-29852, produced an IC_{50} value of 3.56 ± 1.84 mM, whereas SQ-30956 (R-S version of SQ-29852) produced an IC_{50} value of 6.83 ± 0.67 mM. L-Ala-D-Pro had an IC_{50} of 3.97 ± 1.11 mM, however, when the D-amino acid was in the *N*-terminal position (D-alanyl-L-proline {D-Ala-L-Pro}) the affinity was reduced producing an IC_{50} value of 17.49 ± 2.52 mM. Therefore, the effect of changing the stereochemistry of the amino acid (L or D) at the *N*-and *C*-terminals position on the affinity of the dipeptide for the DTS, is dependent upon the amino acid rather than the position of the change (*N*- or *C*-terminal).

Domain C showed a preference for functional groups with no charge (L-norLeu-L-Pro, IC_{50} = 0.11 ± 0.04 mM). Introduction of a negative charge, as in 2-amino-adipyl-L-Pro, or positive charge, as in L-Orn-L-Pro in the C domain of the molecule, resulted in a reduction in affinity, producing IC_{50} values of 0.32 ± 0.06 mM and 0.45 ± 0.10 mM respectively. However, these values were not significantly different indicating that neither anionic nor cationic centres in this area provide specific interactions.

5.1 BACKGROUND

5.1.1 INTRODUCTION

Although some information regarding the stereochemical preference of the DTS is known, there are still questions remaining that need to be resolved. The general opinion is that the DTS has a preference for L- over D-enantiomers, with the D, D dipeptides being poorly absorbed, slowly hydrolysed, but not entirely without affinity for the DTS [Matthews, 1987]. However, other workers have demonstrated that the D, D dipeptides are not substrates for the DTS [Boyd & Ward, 1982: Thwaites *et al.*, 1994a], with the intestinal absorption of D-alanyl-D-phenylalanine (D-Ala-D-Phe) being almost zero in rats [Asatoor *et al.*, 1973]. The inclusion of D-amino acids has been demonstrated to confer a degree of hydrolysis-resistance to peptidase activity [Asatoor *et al.*, 1973, Boyd & Ward 1982], and to reduce the rate of absorption from the intestinal lumen [Asatoor *et al.*, 1973: Bai & Amidon 1992]. The effect of D-enantiomers at the *C*- or *N*-terminal has yet to be fully established. A number of drug absorption studies investigating the DTS have used cephalosporins (*e.g.* cephalexin and cephradine) as the experimental probe. Part of the cephalosporin structure has similarities to the unusual dipeptide, D-Ala-D-Ala. Although the cephalosporins are hydrolysis-resistant due to this D-configuration, they have the disadvantage of reduced affinity for the DTS. Therefore, the aim of this chapter is to investigate the stereochemical preference of the present model system in comparison with other model systems, and to evaluate the use of cephalosporins as probe compounds. The effect on uptake of the probe, by compounds with a negative, positive or neutral charge in domain C is also explored.

5.1.2 STEREOSELECTIVITY OF THE DTS

All studies are in agreement that the transporter has a preference for L-isomers, but the interaction of D, D dipeptides with the transporter is not as clear. Some studies have shown that dipeptides consisting exclusively of D-enantiomers are not substrates for the transporter [Asatoor *et al.*, 1973: Boyd & Ward, 1982: Thwaites *et al.*, 1994a], while others have demonstrated that they are substrates but with reduced affinity [Addison *et al.*, 1975a, b: Wenzel *et al.*, 1995]. Both D- and L-isomers of the peptidomimetic, cephalexin, were found to interact with the oligopeptide transporter in Caco-2 cells [Dantzig & Bergin, 1990], with L-cephalexin displaying a greater affinity for the transporter. In contrast, uptake of cephalexin into rat intestinal BBMV was strictly stereospecific for the D-enantiomer [Tamai *et al.*, 1988: Kramer *et al.*, 1992]. Rabbit peptide transporter (PepT1) cloned into oocytes, has been shown to take up D- and L-enantiomers of cephalosporins, with preference for the L-isomer, confirming the concept of stereoselective substrate recognition [Wenzel *et al.*, 1995]. D-Amino acid residues have also been shown to reduce affinity for the transporter in everted rings of hamster jejunum *in vitro* [Addison *et al.*, 1975a,b]. However, the dipeptide D-Leu-D-Leu was found not to be transported in rat jejunum [Asatoor *et al.*, 1973] or *Necturus Maculosus* intestine [Boyd & Ward, 1982], suggesting that D, D dipeptides are not

substrates. D-Leu-D-Leu and D-Ala-D-Ala both failed to inhibit the intracellular accumulation and transcellular transport of [^{14}C]-Gly-Sar into Caco-2 cell monolayers and also to induce H^+ flow into the cells, suggesting they are not a substrate for the DTS [Thwaites *et al.*, 1994a]. Whether a dipeptide containing exclusively D-enantiomers is transported, appears to be dependent upon the model used and the amino acid composition of the dipeptide.

The affect of D-amino acids at the *C*- or *N*-terminal is somewhat controversial. Hidalgo *et al.*, found that the dipeptide L-Val-L-Val was capable of inhibiting uptake of [^{3}H]cephalexin into Caco-2 cells. Changing the chirality of one of the amino acids (*e.g.* L-valyl-D-valine {L-Val-D-Val} or D-valyl-L-valine {D-Val-L-Val}) had no affect on affinity, suggesting that the DTS in Caco-2 cells has no preference for L- or D-configuration at the *C*- or *N*-termini [Hidalgo *et al.*, 1995]. D-Valyl-D-valine (D-Val-D-Val) failed to inhibit uptake, indicating a marked preference for dipeptides containing at least one L-enantiomer. This group also suggested incorporation of a D-amino acid into the dipeptide to increase metabolic stability without compromising interaction with the DTS [Hidalgo *et al.*, 1995].

It has been shown that incorporation of a D-amino acid into the *N*-terminal of a di- or tripeptide reduces the rate of absorption significantly [Asatoor *et al.*, 1973: Thwaites *et al.*, 1994a]. This was confirmed in a study using Ala peptides and Caco-2 cells. Incorporation of D-amino acids into the *N*-terminal reduced affinity for interaction, although D-amino acids incorporated at the *C*-terminal had a greater affect. This contradicts a previous study, where no preference for L- or D-configuration at the *C*- or *N*-termini was evident [Hidalgo *et al.*, 1995]. D, D dipeptides where found not to be substrates for the DTS [Wenzel *et al.*, 1995] yet Dantzig & Bergin found both L- and D-cephalexin to be substrates [Dantzig & Bergin, 1990]. It has been suggested that orally active β-lactams should have affinities similar to di- and tripeptides with an *N*-terminal D-amino acid, as they all have an unsubstituted or modified D-phenylalanyl-glycine {D-Phe-Gly} side group in the *N*-terminal position [Amidon & Sinko, 1988: 1989]. Aminocephalosporins have been reported to have K_m and K_i values which are similar to values obtained for di and tripeptides, although overall affinity may be reduced due to conformational restraint in the lactam ring system [Ganapathy *et al.*, 1984: Kudo *et al.*, 1989].

The most in-depth study was carried out in isolated rat small intestinal loops, looking at the transport of eight dipeptides (combinations of Phe and Ala) [Lister *et al.*, 1995]. The dipeptide, L-phenylalanyl-L-alanine {L-Phe-L-Ala}, gave the highest rate of transfer, agreeing with the fact that a lipophilic side chain at *N*-terminal enhances transport [Matthews, 1987]. D-Phe in the N-terminal position (D-phenylalanyl-L-alanine {D-Phe-L-Ala}) of the dipeptide reduced the transport rate by 90 %, but when in the *C*-terminal position (L-alanyl-D-phenylalanine {L-Ala-D-Phe}), the effect was minimal. *N*-terminal D-Ala (D-alanyl-L-phenylalanine {D-Ala-L-Phe}) had little effect on transport, but when D-Ala was at the *C*-terminal (L-phenylalanyl-D-alanine {L-Phe-D-Ala}) the rate was cut by 80 %. The dipeptides containing only D-isomers (D-alanyl-D-phenylalanine {D-Ala-D-Phe} and D-

phenyalanyl-D-alanine {D-Phe-D-Ala}) were not absorbed or hydrolysed by the intestine to an appreciable extent [Lister *et al.*, 1995].

Lister *et al.*, have devised four general conclusions regarding peptide absorption;

- The apical membrane transporter is sensitive to the chirality of both amino acids,
- Peptides with *N*-terminal L-Phe are more sensitive to the introduction of a *C*-terminal D-amino acid than the peptides with an *N*-terminal L-Ala,
- The dipeptide transporter at the basolateral membrane has a different specificity from the apical transporter,
- Low permeability of D, D dipeptides indicates that there is minimal paracellular transport of short peptides in the intact rat small intestine [Lister *et al.*, 1995].

5.1.3 THE EFFECT OF CHARGE IN THE C DOMAIN OF A MOLECULE ON INTERACTION WITH THE DTS

Structural activity relationships for the DTS are somewhat limited as, until the present study, no systematic investigation of the structural requirements for peptide transport had been carried out. Very little information is known regarding functional groups which increase affinity for the transporter. Domain C in our proposed structure (Chapter 4, Figure 4.1), corresponds to the *N*-terminal amino acid side-chain. Gly-Pro influx across the intestinal mucosal border of rabbits was only inhibited by dipeptides with a free amino group in the α-position [Rubino *et al.*, 1971]. Dipeptides with an imino or an acetylated amino group in the α-position failed to inhibit flux, suggesting that a free amino group in the α-position is essential for transport [Rubino *et al.*, 1971]. However, a later study using rat intestinal segments and the dipeptide Phe-Pro found that dipeptide analogues without an α-amino group at the *N*-terminal position were able to inhibit transport, contradicting the previous study and indicating that a free α-amino group is not essential for transport [Bai *et al.*, 1991]. The introduction of negative or double positive charges in the amino acid side chains has been shown to greatly decrease affinity for the transporter [Wootton & Hazelwood, 1989], whereas, lipophilic side-chains, such as Phe have been shown to enhance affinity [Matthews, 1987]. Based on studies using photoaffinity probes for the DTS in rabbit BBMV, a hypothetical peptide structure for recognition and transport by the intestinal oligopeptide transporter (DTS) has been proposed (Chapter 4, Figure 4.1) [Kramer *et al.*, 1995]. R_3 of the hypothetical structure which correlates to domain C in the present model is suggested to have some structural variety, but must not be a negatively charged group [Kramer *et al.*, 1995]. The aim of the present experiment is to investigate the effect of charge in the C domain by using a series of dipeptides were the remainder of the molecule is kept constant.

5.2 MATERIALS AND METHODS

5.2.1 MATERIALS

Details of the suppliers of all materials used throughout this chapter have been described previously (section 2.1-2.1.3).

5.2.2 METHODS

5.2.2.1 MEDIA

Details of growth media (M1), and incubation media (M4) used throughout this chapter have been described previously (section 2.2.1.1).

5.2.2.2 CELL CULTURE

Cell line-1 (section 2.1.1) was used between passage numbers 25 to 38. The cells were seeded onto 24-well plates (2 cm^2) at a density of 1.6 x 10^5 cells $well^{-1}$, and grown in an atmosphere of 5 % CO_2 (95 % air) and 90 % relative humidity, at 37°C, as previously described (section 2.2.1.3). M1 was renewed every 48 h and the monolayers were used after 7 d growth, unless otherwise stated.

5.2.2.3 EXPERIMENTAL CONDITIONS FOR UPTAKE STUDIES

The following procedure and conditions were used for all studies unless otherwise stated: All solutions were preincubated at 37°C prior to experimentation, with the exception of PBS-azide which was kept at 4°C. The maintenance media was aspirated and the monolayers were washed with M4 at pH 6 (1 x 500 µl x 5 min) at 37°C. The incubation solution consisted of M4 with 2 % DMSO at pH 6, containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro, and the required amount of competitor (0.1, 0.5, 1 or 10 mM). Monolayers were incubated (1 x 250 µl x 3 min), at 37°C. Apical solutions were then collected, and the plates transferred to a cold table (4°C) and washed with ice-cold PBS-azide solution (2 x 500 µl x 5 min). The monolayers were then harvested by solubilising with 1 ml of 1 % v/v TX-100 in double distilled water. Apical solutions, washings and solubilised cells were collected made up to 1 ml with double distilled water, added to 10 ml HiSafe 3, and counted for ^{3}H content by LSC. Results were corrected for 15 % non-specific uptake, and expressed % inhibition from the control value (Gly-[^{3}H]L-Pro uptake in the absence of competitor) and IC_{50} (mM) (Chapter 3, Equation 3.4). Data are mean value (of at least three monolayers) ± S.D. Competitors which produced inhibition values lower than 15 % or higher than 85 % were retested at a higher or lower concentration respectively, in order to achieve values within this range. As more enantiomers were tested, reliable anticipation of appropriate ranges developed, allowing compounds to be tested at the correct concentration without previous study. All results are shown for competitors which were tested at more than one concentration.

5.2.3 STEREOCHEMICAL PREFERENCE OF THE DTS

5.2.3.1 SQ-29852 ENANTIOMERS

Uptake of Gly-[^{3}H]L-Pro in the presence of the following enantiomers of SQ-29852 were investigated (Table 5.1); SQ-29852 (S, S), SQ-32009 (S, R), SQ-30956 (R, S), and SQ-32389 (R, R). SQ-29852 and SQ-32009 were tested at 1 mM, whereas SQ-30956 and SQ-32389 were tested at 10 mM. Note, that (S, S) peptidomimetic drugs in this instance corresponds to (L, L) dipeptides.

5.2.3.2 LYS-PRO ENANTIOMERS

Uptake of Gly-[^{3}H]L-Pro in the presence of the following Lys-Pro enantiomers were investigated (Table 5.2) at the concentration shown; L-Lys-L-Pro (0.1 mM), L-Lys-D-Pro (1, 5, and 10 mM), D-Lys-L-Pro (10 mM), and D-Lys-D-Pro (10 mM).

5.2.3.3 ALA-PRO ENANTIOMERS

Uptake of Gly-[^{3}H]L-Pro in the presence of the following Ala-Pro enantiomers were investigated (Table 5.3) at the concentration shown; L-alanyl-L-proline (L-Ala-L-Pro) (0.1 and 1 mM), L-Ala-D-Pro (0.1 and 1 mM), D-Ala-L-Pro (1 and 10 mM), and D-Ala-D-Pro (10 mM).

5.2.3.4 ALA-ALA ENANTIOMERS

Uptake of Gly-[^{3}H]L-Pro in the presence of the following Ala-Ala enantiomers were investigated (Table 5.4) at the concentration shown; L-Ala-L-Ala (0.1 and 1 mM), D-Ala-D-Ala (1 and 10 mM), and DL-alanyl-DL-alanine (DL-Ala-DL-Ala) (0.5 and 1 mM).

5.2.4 CHARGE PREFERENCE OF THE DTS FOR DOMAIN C

5.2.4.1 EFFECT OF CHARGE IN DOMAIN C ON INTERACTION WITH THE DTS

Uptake of Gly-[^{3}H]L-Pro in the presence of the following L-Pro-containing-dipeptides were investigated (Table 5.5) at the concentration shown; L-norLeu-L-Pro (0.1, 0.5, 1 and 10 mM), 2-amino-adipyl-L-Pro (1 and 10 mM), and L-Orn-L-Pro (1 and 10 mM).

5.2.5 EFFECT OF SELECTED AMINO ACIDS ON GLY-[^{3}H]L-PRO UPTAKE

5.2.5.1 EFFECT OF THE DIPEPTIDE AMINO ACID COMPONENTS ON GLY-[^{3}H]L-PRO UPTAKE INTO CACO-2 CELL MONOLAYERS

Uptake of Gly-[^{3}H]L-Pro in the presence of the following amino acids was investigated at the concentration shown; L-Lys (1 and 10 mM), D-lysine (D-Lys) (1 and 10 mM), L-Ala (10 mM), D-Ala (10 mM), DL-alanine (DL-Ala) (10 mM), L-norleucine (L-

norLeu) (1 and 10 mM), 2-amino-adipic acid (1 and 10 mM) and L-ornithine (L-Orn) (1 and 10 mM).

5.3 RESULTS AND DISCUSSION

5.3.1 STEREOCHEMICAL PREFERENCE OF THE DTS

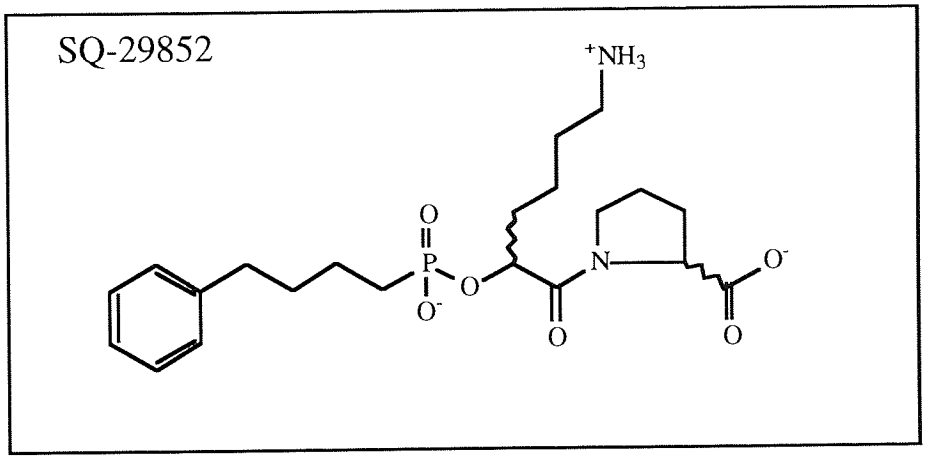
The aim of this chapter was to investigate the stereoselective preference of the DTS, with regard to the peptidomimetic drugs which utilise the transporter to some extent to contribute to their absorption. The compounds used within this chapter were selected for the following reasons; 1) all four enantiomers of the peptidomimetic drug SQ-29852, were available, 2) SQ-29852 and lisinopril are L-Lys-L-Pro analogue, 3) the ACE-inhibitor enalapril, is an L-Ala-L-Pro analogue and 4) the cephalosporins are tripeptide analogues with part of the structure possessing similarities to D-Ala-D-Ala.

5.3.1.1 SQ-29852 ENANTIOMERS

SQ-29852, as previously mentioned, is a stable and specific probe for the DTS [Marino *et al.*, 1996: Nicklin *et al.*, 1996], that has been shown to inhibit Gly-[^{3}H]L-Pro uptake into Caco-2 cells in a competitive manner (section 3.3.2) in the present system. 1 mM SQ-29852 produced 60.51 ± 6.73 % inhibition with a corresponding IC_{50} of 0.68 ± 0.17 mM. In this instance, SQ-29852 is the S, S version of the compound, which is equivalent to an L, L dipeptide. Changing the stereochemistry at the *C*-terminal (SQ-32009) produced a reduction in affinity for the transporter (Table 5.1), with 1 mM producing 21.14 ± 8.22 % inhibition. Statistical analysis comparing the results to the control compound (SQ-29852) could not be carried out using the inhibition data for all four enantiomers, as different concentrations of competitor were used. Therefore, statistical analysis were carried out on the IC_{50} values, as this calculation corrects for concentration. IC_{50} values for SQ-29852 (S, S) and SQ-32009 (S, R), 0.68 ± 0.17 mM and 3.56 ± 1.84 mM respectively, were not significantly different from one another at the confidence limits tested (95 %), probably due to the large S.D. of SQ-32009 resulting from conversion into an IC_{50} value. When the chirality at the *N*-terminal is changed from S to R (SQ-30956, R, S) the IC_{50} value increased to 6.83 ± 0.67, which corresponds to a reduction in recognition. Note that SQ-30956 (R, S) and SQ-32389 (R, R) were tested at 10 mM. SQ-32389 (R-R) which is analogous to a D, D dipeptide, again showed a reduction in recognition compared to SQ-29852 (S, S) and SQ-32009 (S, R). SQ-30956 and SQ-32389 produced values which were not significantly different at the confidence limits (95 %) tested. These results suggest that the stereochemistry at the *N*-terminal may be more important for recognition than that at the *C*-terminal. However, the IC_{50} values for SQ-32009 (S, R), SQ-30956 (R, S) and SQ-32389 (R, R) are within the same range, with the values for SQ-32389 (R, R) and SQ-32009 (S, R) not quite being significantly different at the confidence limits (95 %) tested. Thus, in future experiments, SQ-32009 (S, R) should be tested at 10 mM and therefore, directly comparable

to SQ-30956 (R, S) and SQ-32389 (R, R) . However, the results clearly illustrate that the S, S version (SQ-29852) is the most preferred enantiomer, agreeing with previous literature [Matthews, 1987: Thwaites *et al.*, 1994a], with the R, R version (SQ-32389) being recognised but displaying reduced affinity when compared to S, S.

TABLE 5.1 *Preference of the DTS for SQ-29852 enantiomers*



COMPOUND	Conc (mM)	Stereochemistry of bonds *N*-terminal	*C*-terminal	Inhibition % Mean [S.D.]	IC_{50} mM Mean [S.D.]
SQ-29852	1	S	S	60.51 [6.73]	0.68 [0.17]
SQ-32009	1	S	R	24.14 [8.22]	3.56 [1.84]
SQ-30956	10	R	S	59.49 [2.44]	6.83 [0.67][a]
SQ-32389	10	R	R	61.11 [2.39]	6.38 [0.63][a]

Monolayers were washed (1 x 500 µl x 5 min) with M4. They were then incubated (1 x 250 µl x 3 min) at 37°C, with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6 and in the presence of the competitor (for concentration see table). Data are corrected for 15 % non-specific uptake and presented as mean % inhibition ± S.D. for three monolayers and mean IC_{50} ± S.D. mM, with [a] denoting significant inhibition at $p \leq 0.0001$ from the control value (uptake of Gly-[^{3}H]L-Pro in the presence of 1 mM SQ-29852).

5.3.1.2 LYS-PRO ENANTIOMERS

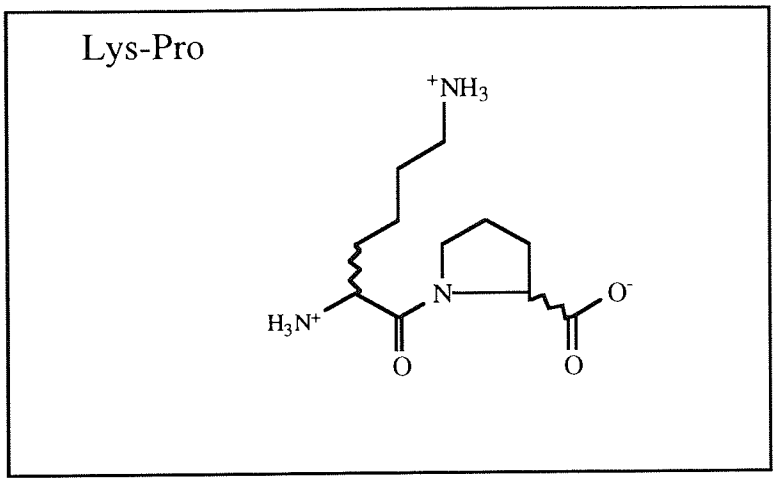
The L-Lys-L-Pro enantiomer produced the lowest IC_{50} value (0.22 ± 0.01 mM) and, therefore, had the greatest affinity for the transporter compared to the other enantiomers (Table 5.2). L-Lys-L-Pro showed significantly greater affinity for the transporter than SQ-29852, which is an analogue of the dipeptide. This is not unexpected as one would anticipate that the dipeptide being a natural substrate, would display better affinity for the DTS. Changing the amino acid at the *C*-terminal to a D-enantiomer (L-Lys-D-Pro) reduced affinity for the DTS, producing an IC_{50} of 29.94 ± 3.18 mM. However, when the D-enantiomer was at the *N*-terminal position (D-Lys-L-Pro) the effect was not as dramatic (IC_{50} = 12.54 ± 0.61 mM). This contradicts previous data shown for the SQ-29852 enantiomers (Table 5.1), which found that an R-enantiomer (D) at the *N*-terminal produced a greater reduction in affinity than when at the *C*-terminal. The IC_{50} values for L-Lys-D-Pro and D-Lys-D-Pro, 29.94 ± 3.18 mM and 30.86 ± 4.07 mM respectively are not significantly different at the confidence limits (95 %) tested. Again, the D, D version still shows affinity for the transporter, disagreeing with literature that states that D-D dipeptides are not substrates for the DTS [Asatoor *et al.*, 1973: Boyd & Ward 1982: Thwaites *et al.*, 1994a]. L-Lys-L-Pro has a greater affinity for the DTS than its analogue SQ-29852. However, the other Lys-Pro enantiomers tested all show lower affinities than the corresponding SQ-29852 analogues, suggesting that stereochemistry plays a greater part in uptake of dipeptides compared to peptidomimetic drugs, which appear to be more tolerant to stereochemical alterations. However, the stereochemical changes with the SQ-29852 analogues may be swamped by other substituent effects.

5.3.1.3 ALA-PRO ENANTIOMERS

L-Ala-L-Pro produced 52.11 ± 6.63 % inhibition giving an IC_{50} of 0.09 ± 0.02 mM at a 0.1 mM concentration (Table 5.3). Changing the chirality of the amino acid at the *C*-terminal to a D-enantiomer produced a significant reduction in affinity, giving an IC_{50} of 3.97 ± 1.11 mM. However, the reduction seen in affinity when the D-amino acid is at the *N*-terminal (D-Ala-L-Pro) is much greater, giving an IC_{50} of 17.49 ± 2.52 mM. Interestingly, D-Ala-D-Pro produced the highest IC_{50} value (27.92 ± 6.18 mM) therefore having the lowest affinity for the DTS. For the two previous series of enantiomers, SQ-29852 and Lys-Pro, the D, D version had approximately the same affinity as the D, L and L, D isomers respectively, which disagrees with previous findings that the D, D dipeptides have reduced affinity compared to other combinations of enantiomers [Hidalgo *et al.*, 1995]. This is the first series to demonstrate that the D, D dipeptide has reduced affinity compared to the other enantiomers, in agreement with previous work [Addison *et al.*, 1975a, b]. However, the IC_{50} values for D-Ala-L-Pro and D-Ala-D-Pro are not quite significantly different at the 95 % confidence limits tested and, therefore, retesting the compounds at a lower concentration (*e.g.* 5 mM), may allow the compounds to be ranked with regard to affinity

Interestingly, the IC_{50} value for D-Ala-D-Pro is not significantly different from those for D-Lys-D-Pro and L-Lys-D-Pro.

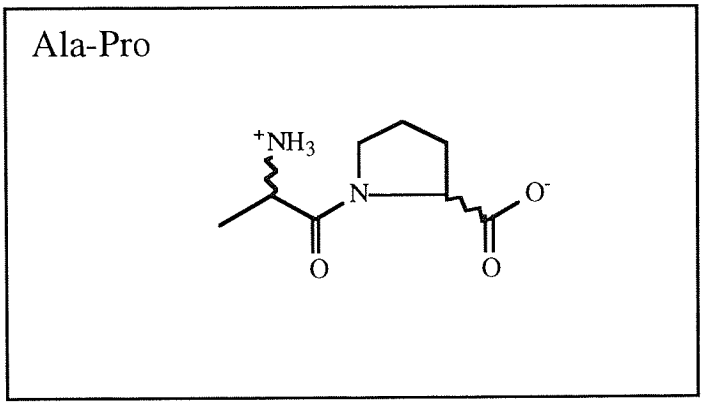
TABLE 5.2 *Preference of the DTS for Lys-Pro enantiomers*



COMPOUND Enantiomer	Conc (mM)	Inhibition % Mean [S.D.]	IC_{50} mM Mean [S.D.]
L-Lys-L-Pro	0.1	30.94 [0.92]^{c}	0.22 [0.01]
L-Lys-D-Pro	1	1.31 [7.32]	y
	5	12.61 [2.89]^{c}	36.11 [9.73]
	10	31.51 [3.07]^{a}	29.94 [3.18]
D-Lys-L-Pro	10	44.39 [1.19]^{c}	12.54 [0.61]
D-Lys-D-Pro	10	24.63 [2.32]^{b}	30.86 [4.07]

Monolayers were washed (1 x 500 µl x 5 min) with M4. They were then incubated (1 x 250 µl x 3 min) at 37°C, with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6 and in the presence of the competitor (for concentration see table). Tests were repeated using a higher or lower concentration of compound to achieve inhibition between 15-85 %. Data are corrected for 15 % non-specific uptake and presented as mean % inhibition ± S.D. for three monolayers and mean IC_{50} ± S.D. mM, with ^{a}, ^{b} and ^{c} denoting significant inhibition at $p < 0.001$, $p < 0.005$ and $p < 0.01$, respectively, from the control value (uptake of Gly-[^{3}H]L-Pro in the absence of competitor). y corresponds to a % inhibition value which is too small (% inhibition ≤ 0.00) to be converted into a relevant IC_{50} value.

TABLE 5.3 *Preference of the DTS for Ala-Pro enantiomers*



COMPOUND Enantiomer	Conc (mM)	Inhibition % Mean [S.D.]	IC_{50} mM Mean [S.D.]
L-Ala-L-Pro	1	99.42 [0.73]^{a}	0.006 [0.007]
	0.1	52.11 [6.63]^{b}	0.09 [0.02]
L-Ala-D-Pro	1	20.92 [5.32]^{d}	3.97 [1.11]
D-Ala-L-Pro	1	7.32 [2.44]^{d}	13.58 [4.13]
	10	36.57 [3.20]^{b}	17.49 [2.52]
D-Ala-D-Pro	10	26.90 [4.83]^{c}	27.92 [6.18]

Monolayers were washed (1 x 500 µl x 5 min) with M4. They were then incubated (1 x 250 µl x 3 min) at 37°C, with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6 and in the presence of the competitor (for concentration see table). Tests were repeated using a higher or lower concentration of compound to achieve inhibition between 15-85 %. Data are corrected for 15 % non-specific uptake and presented as mean % inhibition ± S.D. for three monolayers and mean IC_{50} ± S.D. mM, with ^{a}, ^{b}, ^{c} and ^{d} denoting significant inhibition at $p < 0.0001$, $p < 0.001$, $p < 0.01$ and $p < 0.05$ respectively, from the control value (uptake of Gly-[^{3}H]L-Pro in the absence of competitor).

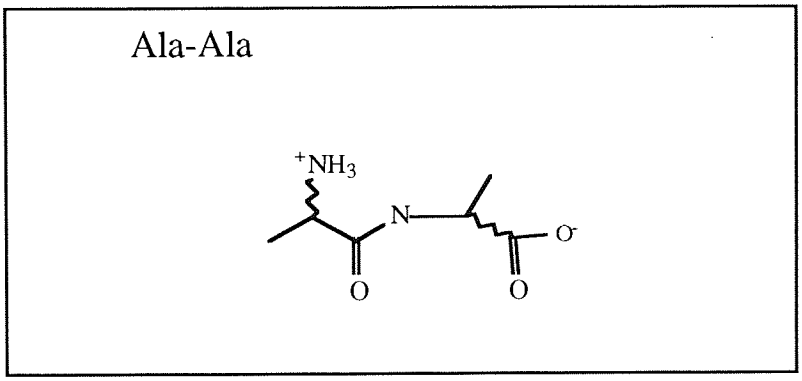
5.3.1.4 ALA-ALA ENANTIOMERS

L-Ala-L-Ala at a concentration of 0.1 mM gave an IC_{50} of 0.09 ± 0.02 mM (Table 5.4), which is the same as for L-Ala-L-Pro, illustrating that the Pro ring is not required for recognition. D-Ala-D-Ala produced no significant reduction from the control (Gly-[^{3}H]L-Pro uptake in the absence of competitor) at the concentrations tested (1 and 10 mM). This is in agreement with reports that D-D dipeptides are not substrates for the DTS [Asatoor *et al.*, 1973: Boyd & Ward, 1982]. However, it contradicts the finding that both L- and D-cephalexin (part of structure is analogous to that of D-Ala-D-Ala) are substrates for the DTS [Dantzig *et al.*, 1990] and that cephalexin uptake into rat intestinal BBMV is stereospecific for the D-enantiomer [Tamai *et al.*, 1988: Kramer *et al.*, 1992]. The mixed enantiomer, DL-Ala-DL-Ala, produced 51.85 ± 3.32 % inhibition, which can be attributed to the L-L and possibly the L-D and D-L isomers in the mixture, which may have affinity for the DTS.

5.3.2 THE EFFECT OF CHARGE IN THE C DOMAIN ON UPTAKE OF GLY-[^{3}H]-L-PRO INTO CACO-2 CELL MONOLAYERS

The effect of charge in the C domain on interaction with the DTS was assessed by co-administering the probe with a series of dipeptides (Table 5.5) possessing the same basic structure, having a neutral, positive or a negative charge in the C domain of the molecule based on the previous domain allocation (Chapter 4, Figure 4.1). L-norLeu-L-Pro which had no charge in the C domain, and therefore, is a zwitterion, displayed the greatest affinity for the DTS, producing an IC_{50} value of 0.11 ± 0.04 mM. When a carboxylic acid group is substituted, giving the domain C region of the molecule a negative charge, as with 2-amino-adipyl-L-Pro, the affinity for the transporter is significantly reduced, producing an IC_{50} value of 0.32 ± 0.06 mM. L-Orn-L-Pro which has an amine group in the C domain, giving the region a positive charge, the affinity is again reduced further (IC_{50} = 0.45 ± 0.10 mM). % inhibition and IC_{50} values for 2-amino-adipyl-L-Pro and L-Orn-L-Pro are not significantly different at the confidence limits (95 %) tested. These results indicate that the DTS has a preference for the functional group in the C domain to have no charge. Compounds with a negative or positive charge in this region show reduced affinity, but the transporter appears to have no preference over positive or negative charges. These findings disagree with a proposed hypothetical structure designed for uptake by the peptide transporter, which states that R_3 (corresponding to the C domain) must not be a negatively charged group [Kramer *et al.*, 1995]. The present study clearly illustrates that although a negative charge reduces affinity as seen with 2-amino-adipyl-L-Pro, the compound is still a substrate for the DTS in the present system. This was previously illustrated in Chapter 4, where SQ-13926 and *N*-succinyl-L-proline, which both possess negative charges in domain C still showed affinity for the DTS, with IC_{50} values of 6.36 ± 1.68 mM and 3.88 ± 1.35 mM respectively.

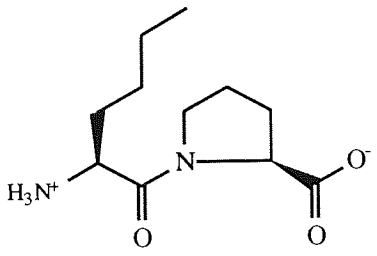
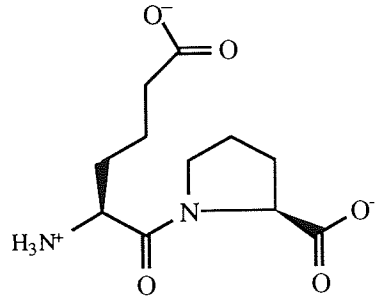
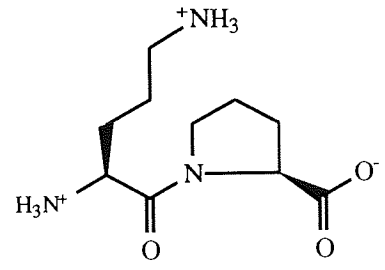
TABLE 5.4 *Preference of the DTS for Ala-Ala enantiomers*



COMPOUND Enantiomer	Conc (mM)	Inhibition % Mean [S.D.]	IC_{50} mM Mean [S.D.]
L-Ala-L-Ala	1	102.27 [0.98][a]	x
	0.1	52.90 [4.35][a]	0.09 [0.02]
D-Ala-D-Ala	1	-4.13 [4.74]	y
	10	2.91 [7.90]	y
DL-Ala-DL-Ala	1	85.24 [1.72][a]	0.17 [0.02]
	10	51.85 [3.32][a]	0.47 [0.06]

Monolayers were washed (1 x 500 µl x 5 min) with M4. They were then incubated (1 x 250 µl x 3 min) at 37°C, with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6 and in the presence of the competitor (for concentration see table). Tests were repeated using a higher or lower concentration of compound to achieve inhibition between 15-85 %. Data are corrected for 15 % non-specific uptake and presented as mean % inhibition ± S.D. for three monolayers and mean IC_{50} ± S.D. mM, with [a] denoting significant inhibition at $P \leq 0.0001$, from the control value (uptake of Gly-[^{3}H]L-Pro in the absence of competitor). x and y correspond to % inhibition values which are too large (% inhibition ≥ 100.00) or too small (% inhibition ≤ 0.00) respectively, to be converted into relevant IC_{50} values.

TABLE 5.5 *Preference of charge by the DTS in the C domain*

COMPOUND	Conc (mM)	Structure	Inhibition % Mean [S.D.]	IC_{50} mM Mean [S.D.]
L-norLeu-L-Pro	10		100.72 [1.87]^{c}	x
	1		88.46 [3.56]^{a}	0.13 [0.05]
	0.5		77.17 [1.67]^{c}	0.15 [0.01]
	0.1		48.20 [7.77]c	0.11 [0.04]
2 amino adipyl-L-Pro	10		92.13 [3.00]^{a}	0.86 [0.36]
	1		76.05 [3.58]^{b}	0.32 [0.06]
L-Orn-L-Pro	10		87.54 [2.13]^{a}	1.43 [0.28]
	1		69.15 [4.68]^{b}	0.45 [0.10]

Monolayers were washed (1 x 500 µl x 5 min) with M4. They were then incubated (1 x 250 µl x 3 min) at 37°C, with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6 and in the presence of the competitor (for concentration see table). Tests were repeated using a higher or lower concentration of compound to achieve inhibition between 15-85 %. Data are corrected for 15 % non-specific uptake and presented as mean % inhibition ± S.D. for three monolayers and mean IC_{50} ± S.D. mM, with ^{a}, ^{b} and ^{c} denoting significant inhibition at $p < 0.0001$, $p < 0.0005$ and $p < 0.01$ respectively, from the control (uptake of Gly-[^{3}H]L-Pro in the absence of competitor). x corresponds to a % inhibition value which is too large (% inhibition ≥ 100.00) to be converted into a relevant IC_{50} value.

5.3.3 THE EFFECT OF A SERIES OF SELECTED AMINO ACIDS ON GLY-[^{3}H]L-PRO UPTAKE INTO CACO-2 CELL MONOLAYERS

The series of amino acids investigated (Figure 5.1) were selected because they are components of the dipeptides tested previously. It was necessary to ensure that the inhibitory effect observed with the dipeptides, was a result of the dipeptides and not amino acid residues arising from hydrolysis. SQ-29852 is a stable compound [Marino *et al.*, 1996], and dipeptides containing Pro possess a degree of resistance to hydrolysis [Matthews, 1987]. Ala-Ala enantiomers maybe subject to hydrolysis at the apical membrane of the cells, however, the short incubation period of 3 min, will minimise degradation due to hydrolysis if present. All the amino acids tested failed to significantly inhibit the probe at the confidence limits (95 %) tested, indicating that the results observed previously are due to the dipeptides and not their amino acid components. The Pro enantiomers were not included in the study, as previously, (Chapter 3, section 3.3.1) L-Pro has been shown to produce significant inhibition of Gly-[^{3}H]L-Pro uptake. The inhibition seen with L-Pro can be attributed to inhibition of [^{3}H]L-Pro (produced by hydrolysis of the Gly-[^{3}H]L-Pro) which is then taken up into the cells *via* the active imino-acid carrier system [Nicklin *et al.*, 1992a]. Therefore, 10 mM L-Pro is included in all experimental solutions to block any [^{3}H]L-Pro uptake into the Caco-2 cell monolayers. All the amino acids tested failed to significantly inhibit the probe at the confidence limits (95 %) tested, indicating that the results observed previously are due to the dipeptides and not their amino acid components.

FIGURE 5.1 *Structures of the selected amino acids*

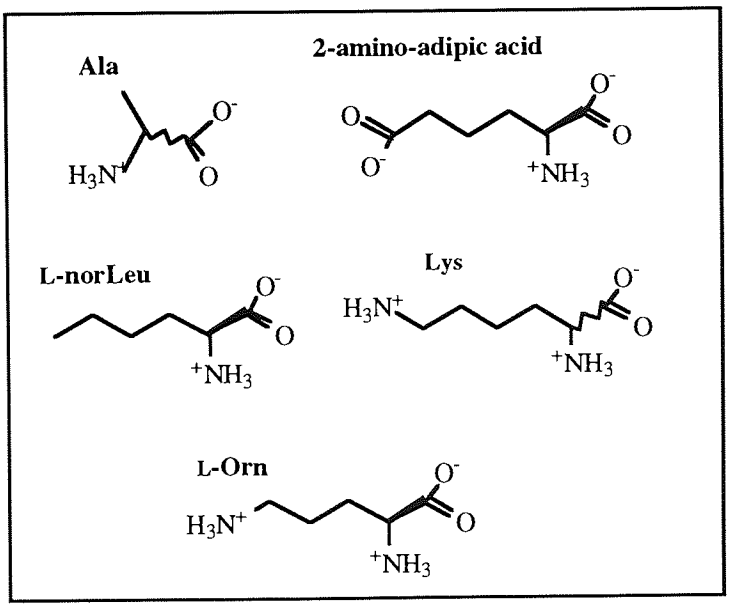


TABLE 5.6 *The effect of a series of amino acids on Gly-[^{3}H]L-Pro uptake*

Compound	Stereochemistry L or D form	Conc (mM)	Inhibition % Mean [S.D.]	IC_{50} mM Mean [S.D.]
Lys	L	1	2.27 [3.97]	y
	L	10	1.38 [3.52]	y
	D	1	-3.34 [5.90]	y
	D	10	3.34 [5.86]	y
Ala	L	10	-3.41 [6.16]	y
	D	10	-7.44 [3.21]	y
	DL	10	3.37 [8.52]	y
2-amino-adipic acid	L	1	-2.46 [8.55]	y
		10	-3.42 [4.52]	y
norLeu	L	1	3.00 [16.09]	y
		10	-13.49[10.7]	y
Orn	L	1	-14.79[12.5]	y
		10	9.03 [0.934]	101.45 [10.91]

Monolayers were washed (1 x 500 µl x 5 min) with M4. They were then incubated (1 x 250 µl x 3 min) at 37°C, with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6 and in the presence of the competitor (for concentration see table). Tests were repeated using 10 mM of competitor to ensure no interaction with the DTS. Data are corrected for 15 % non-specific uptake and presented as mean % inhibition ± S.D. for three monolayers and mean IC_{50} ± S.D. mM. y corresponds to a % inhibition value which is too small (% inhibition ≤ 0.00) to be converted into a relevant IC_{50} value.

5.4 CONCLUSION

The present study illustrates that for all compounds tested the L, L enantiomers displayed the greatest affinity for the transporter as previously shown [Hidalgo *et al.*, 1995: Lister *et al.*, 1995]. The position of the D-amino acid (R) in SQ-29852 enantiomers was found to have a greater effect at the *N*-terminal than at the *C*-terminal, which is in agreement with results for D-Phe-L-Ala transport [Lister *et al.*, 1995]. However, for Lys-Pro enantiomers, the D-amino acid at the *C*-terminal position had a greater reduction on Gly-[^{3}H]L-Pro uptake. The D, D isomers of both SQ-29852 and Lys-Pro were not without affinity for the transporter. The concept that D, D isomers display the lowest affinity is not shown in the present system. Enantiomers of D, L and L, D in some cases (SQ-29852 and Lys-Pro analogues) produced the same results as those for the D, D isomers. L-Ala-L-Pro and L-Ala-L-Ala had a greater affinity for the DTS compared to L-Lys-L-Pro, suggesting that the Pro ring and the carbon chain in the C domain are not required for recognition. For the Ala-Pro enantiomers, the D-amino acid at the *N*-terminal had a greater affect than when at the C-terminal agreeing with the results for SQ-29852 isomers. D-Ala-D-Ala was the only dipeptide compound tested that failed to inhibit uptake. This questions the suitability of the cephalosporins as probe compounds, due to the similarities between the two structures (cephalosporins and D-Ala-D-Ala). As cephalosporins have been shown to interact with the peptide transporter in Caco-2 cells [Dantzig & Bergin, 1990], the present results suggest that the cephalosporins may be absorbed *via* two transport systems or that there are two different binding sites on the same peptide carrier [Hu *et al.*, 1996]. Domain C demonstrated a clear preference for compounds with no charge in this region (L-norLeu-L-Pro). The presence of a negative or positive charge in this area reduced activity. However, no preference over the charges were shown, contradicting a hypothetical peptide structure for the DTS, which suggests that a negative charge in this area is not tolerated [Kramer *et al.*, 1995]. All the amino acids tested, failed to inhibit uptake of the probe, indicating that the inhibitory effect of the dipeptides in not due to the amino acid components. The conclusion reached, is that L, L isomers display the greatest affinity, whereas, D, D have reduced affinity although not necessarily lower than other isomers. There is no general rule governing the importance of the position of the D-amino acid in mixed isomers, as the effect seems dependent upon the composition of the dipeptide. Further information, gained through intensive studies using a wide variety of dipeptide enantiomers is needed, in order to suggest a possible dipeptide with optimum interaction for the DTS.

CHAPTER 6

THE EFFECT OF PEPTIDOMIMETIC DRUGS ON THE UPTAKE OF GLY-[^{3}H]L-PRO INTO CACO-2 CELL MONOLAYERS

ABSTRACT

All of the ACE-inhibitors tested produced some degree of inhibition of the probe, covering IC_{50} values ranging from 0.11 to 32.25 mM. Generally, prodrugs exhibited a higher affinity for the DTS when compared to their active moieties, which is thought to reflect their higher distribution coefficients. The ACE-inhibitor fosinopril showed the highest affinity for the transporter, with an IC_{50} value of 0.11 ± 0.01 mM. This compound has a cyclohexyl group at position 4 of the proline ring, which has previously been shown to increase affinity for the DTS (Chapter 4, section 4.3.3). The ACE-inhibitor benazeprilat, which was thought to be absorbed by a passive process, produced an IC_{50} value of 5.02 ± 1.45 mM, indicating interaction with the DTS. Further investigation is needed to distinguish whether certain compounds are substrates for the DTS, and thus undergo transport into the cell, or are simply blocking the active site of the transporter.

The presence of a lipophilic group adjacent to the amide nitrogen at the *C*-terminus increases affinity with the DTS for ACE-NEP inhibitors. A group of compounds indicated a correlation between lipophilicity of the substituted group and interaction with the DTS. Substitutions, which for certain compounds have been shown to increase affinity, such as the presence of a thiol function and stereochemistry at the *N*-terminus, do not have the same influence for all compounds tested. This illustrates that multiple recognition sites on a molecule may override the deleterious affect of any one change. Molecular modelling of four ACE-NEP inhibitors revealed that the three carbonyls present could be oriented in a similar direction and may be involved in hydrogen bond formation with the active site of the DTS.

The cephalosporins displayed a higher affinity for the DTS compared to the penicillins tested, IC_{50} values of 4.77 - 20.7 mM compared to 39.0 - 44.5 mM respectively. The orally absorbed cephalosporins (cefaclor, cephradine and cephalexin) showed much higher affinity for the transporter compared to the parenteral agent, cefazolin. The majority of the ACE-inhibitors and the β-lactam antibiotics exhibited considerably lower affinity (IC_{50} values of 0.11 ± 0.01 - 44.5 ± 12.9 mM) for the DTS than dipeptides (IC_{50} values of 0.10 ± 0.02 - 3.51 ± 0.97 mM), which are the natural substrates.

6.1 BACKGROUND

6.1.1 INTRODUCTION

The involvement of the DTS in the absorption of some of the ACE-inhibitors [Hu & Amidon, 1988: Friedman & Amidon, 1989a, b: Hu *et al.*, 1995: Thwaites *et al.*, 1995: Nicklin *et al.*, 1996] and β-lactam antibiotics [Nakashima *et al.*, 1984a, b: Okano *et al.*, 1986a, b: Dantzig & Bergin, 1990, 1992, 1994: Gochoco *et al.*, 1994] has been recognised to contribute to their oral bioavailability. Previous investigations have used only a small number of ACE-inhibitors or β-lactam antibiotics in competition studies to access the impact of certain structural features on interaction with the DTS. The competitors are usually tested at a high concentration (10-20 mM) which may well saturate the transporter, thus preventing the affinities of the compounds being ranked with respect to their degree of interaction. The aim of the present chapter is to rank a series of ACE-inhibitors and β-lactam antibiotics for interaction with the DTS and also to compare results with previous studies and clarify the uptake mechanisms utilised.

The peptidomimetic ACE-NEP inhibitors were also selected as potential competitors. These drugs are dual metalloprotease inhibitors, and act by enhancing the action of atrial natriuretic peptide (ANP), which induces diuresis, natriuresis and vasodilatation [Needleman *et al.*, 1989], through the inhibition of NEP and also by inhibiting ACE [Delaney *et al.*, 1994: Robl *et al.*, 1994a, 1994b: Das *et al.*, 1994: Slusarchyk *et al.*, 1995]. Thus simultaneous inhibition of both ACE and NEP offers a useful approach for treating cardiovascular diseases. The ACE-NEP inhibitors are relatively new compounds, and as of yet no information regarding oral bioavailability has been published.

6.1.2 ACE-INHIBITOR ABSORPTION IN CACO-2 CELL MONOLAYERS

The number of investigations looking at ACE-inhibitor absorption/transport in Caco-2 cells is somewhat sparse, as the majority of previous studies have involved animal models rather than cell culture systems. The characteristics of SQ-29852 absorption and transport across Caco-2 cell monolayers have been discussed previously (section 4.1.3).

The apical-to-basolateral transport of lisinopril across Caco-2 cells was found to be lower than that of the paracellular marker mannitol, suggesting a paracellular process [Thwaites *et al.*, 1995]. Transport was also unaffected by lowering the apical pH from pH 7.4 to pH 5.5 and by excess unlabelled lisinopril (20 mM), indicating a pH- and concentration-independent process [Thwaites *et al.*, 1995]. Lisinopril inhibited [^{14}C]-Gly-Sar transport across Caco-2 cell monolayers and steady-state accumulation in Caco-2 cell monolayers. However, lisinopril failed to inhibit the initial uptake of [^{14}C]-Gly-Sar [Thwaites *et al.*, 1995]. Dipeptide *(e.g.* Gly-Sar) transport is associated with H^+ flow into the cell, which results in acidification of the intracellular environment [Thwaites *et al.*, 1993a, b, c, g, 1994a]. However, lisinopril (20 mM) failed to induce a significant change in the intracellular pH of the cell when superfused at the apical membrane, suggesting that

absorption in Caco-2 cell monolayers is *via* a process independent of the DTS [Thwaites *et al.*, 1995]. The results suggest that lisinopril is absorbed *via* a passive process, this is contradictory to previous studies, which have shown lisinopril to have an active uptake component *via* the DTS in rat jejunum [Friedman & Amidon, 1989a: Swaan *et al.*, 1995]. The fact that lisinopril was capable of inhibiting transport and steady-state accumulation of Gly-Sar, suggests that although not a substrate for the DTS in Caco-2 cells, it is capable of blocking the transporter site, and thus preventing/inhibiting interaction with the probe.

Enalapril, at a concentration of 1 mM, failed to inhibit uptake of Gly-[^{3}H]L-Pro into Caco-2 cell monolayers [Morrison *et al.*, 1996]. However, at 10 mM concentration, 33.32 ± 2.21 % inhibition was seen. The authors suggest that the inhibition seen may be due to non-specific effects, resulting from the high concentration of enalapril used [Morrison *et al.*, 1996]. However, enalapril may well have a low affinity for the transporter, and therefore, at 1 mM is without effect, but 10 mM may well exceed its K_i value resulting in inhibition. Enalapril has been shown to inhibit [^{14}C]-Gly-Sar transport and uptake in Caco-2 cells, suggesting that it is competing for the transporter [Thwaites *et al.*, 1995]. Enalapril decreased the pH of the intracellular environment in Caco-2 cells, when superfused at the apical surface, providing evidence for H^+ flow into the cell, consistent with the DTS/H^+ cotransport system [Thwaites *et al.*, 1995]. Interestingly, enalapril caused an increase in the accumulation of the imino acid L-Pro in Caco-2 cells, doubling the uptake of L-Pro compared to the control. A possible explanation is that it may have a high affinity for the imino acid basolateral transporter, and therefore, inhibits the efflux of L-Pro across the basolateral membrane of the cell [Thwaites *et al.*, 1995].

The ACE-inhibitor captopril was capable of inhibiting [^{14}C]-Gly-Sar transport and initial uptake but not uptake at steady-state [Thwaites *et al.*, 1995]. This suggests that captopril may have an inhibitory effect on the basolateral efflux mechanism of the dipeptide or have a higher affinity for the transporter than [^{14}C]-Gly-Sar [Thwaites *et al.*, 1995]. Captopril also caused acidification of the intracellular environment consistent with uptake *via* the DTS/H^+ cotransport system [Thwaites *et al.*, 1995].

Quinapril transport in Caco-2 cells was found to be sensitive to the presence of a H^+ gradient [Hu *et al.*, 1995]. Increasing the apical pH from pH 6 to pH 7.4 resulted in a decrease (15-fold) in quinapril transport, indicating a strong pH-dependence [Hu *et al.*, 1995]. When the apical and basolateral phase were at pH 6, transepithelial transport was slower, suggesting that the transepithelial proton gradient is providing the driving force for transport [Hu *et al.*, 1995]. This was supported by the fact that the protonophore carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), inhibited uptake [Hu *et al.*, 1995]. The protein-modifying agent DEPC and the protein kinase C inhibitor, staurosporin which have both previously been shown to inhibit the DTS [Kramer *et al.*, 1993: Brandsch *et al.*, 1994], inhibited quinapril uptake, suggesting it is a substrate for the DTS [Hu *et al.*, 1995]. Quinapril transport was found to be more sensitive to temperature change than cephalexin transport in Caco-2 cells [Hu *et al.*, 1995]. However, cephalexin transport was

more sensitive to concentration change, suggesting that quinapril has a lower affinity for the transporter [Hu *et al.*, 1995]. The transport of quinapril across Caco-2 cell monolayers was found to be more sensitive to inhibition by peptides and peptide analogues compared to uptake and accumulation, indicating that the inhibitors are acting mainly on the basolateral transport system [Hu *et al.*, 1995]. As only 55 % of quinapril transport was inhibitable, it can be concluded that quinapril transport is *via* a carrier-mediated mechanism with contribution from a nonsaturable process [Hu *et al.*, 1995].

6.1.3 β-LACTAM ANTIBIOTIC ABSORPTION IN CACO-2 CELL MONOLAYERS

Studies investigating β-lactam antibiotic absorption/transport in Caco-2 cells have been carried out due to the fact that the cephalosporins are probes which are relatively stable to hydrolysis as they have structural similarities to the dipeptide D-Ala-D-Ala.

D-[9-^{14}C]Cephalexin accumulated in Caco-2 cells against a concentration gradient [Dantzig & Bergin, 1990]. Uptake was found to be maximal when extracellular pH was 6.0, and reduced by metabolic inhibitors and protonophores [Dantzig & Bergin, 1990]. These results indicate that cephalexin is taken up into Caco-2 cells *via* an energy and proton-dependent process [Dantzig & Bergin, 1990]. Kinetic analysis revealed that cephalexin uptake was composed of a saturable active transport system with a K_m of 7.5 ± 2.8 mM and a V_{max} of 6.5 ± 0.9 nmol min^{-1} mg protein^{-1} [Dantzig & Bergin, 1990]. A non-saturable process with a k_d of 0.18 ± 0.01 nmol min^{-1} mg protein^{-1}, also contributes to the overall transport process [Dantzig & Bergin, 1990]. Uptake was inhibitable by dipeptides, but not by amino acids, and exhibited a stereochemical preference for the L-isomer, thus suggesting uptake is *via* the DTS [Dantzig & Bergin, 1990]. The uptake of cephalexin by a pH-dependent, carrier-mediated process was confirmed in an independent study investigating transepithelial transport [Gochoco *et al.*, 1994].

Transepithelial transport of cephalexin across Caco-2 cells was found to be pH-dependent, with maximum transport being seen when the apical pH was at 6.0 and the basolateral pH was at 7.4 [Gochoco *et al.*, 1994]. Transport was also found to be concentration- and temperature-dependent, suggesting a transcellular process involved in transepithelial transport of cephalexin [Gochoco *et al.*, 1994]. K_t values for uptake and transepithelial transport were 2.9 mM and 4.7 mM respectively, which are comparable to the value (7.5 ± 2.8 mM) reported for uptake, previously [Dantzig & Bergin, 1990]. However, 40 % of transepithelial transport is pH-independent, which is most likely to be a passive process [Gochoco *et al.*, 1994]. Gly-L-Pro and Pro-L-Gly inhibited cephalexin uptake and transport indicating the transepithelial transport is *via* the DTS [Gochoco *et al.*, 1994].

Benzylpenicillin has been reported to have no carrier-mediated uptake [Ryan & Smith, 1989]. However, the K_i of benzylpenicillin was similar to that of cephalexin, 5.5 mM compared to 5.8 mM, respectively [Gochoco *et al.*, 1994]. Benzylpenicillin uptake in Caco-2 cells was found to be minimal and pH-independent, supporting the idea that uptake is not *via* the DTS [Gochoco *et al.*, 1994]. This demonstrates that benzylpenicillin is

capable of binding to the apical transporter site, but this does not result in cellular uptake [Gochoco *et al.*, 1994: Eddy *et al.*, 1995].

Cefaclor, which is a close structural analogue to cephalexin, was found to be taken up into Caco-2 cells *via* a Na^+-independent, energy- and H^+-dependent process [Dantzig *et al.*, 1992], which is consistent with a previous study [Okano *et al.*, 1986a]. Uptake was mediated by a single saturable process with a K_m of 7.6 mM and a V_{max} of 7.6 nmol min^{-1} mg $protein^{-1}$, and a non-saturable process, with a k_d estimated at 0.09 ± 0.02 nmol min^{-1} mg $protein^{-1}$ mM^{-1} [Dantzig *et al.*, 1992]. Cefaclor has the same affinity for the transporter as cephalexin ($K_m = 7.5 \pm 2.8$ mM) in Caco-2 cells [Dantzig & Bergin, 1990]. Uptake was inhibited by dipeptides, orally absorbed antibiotics and to some extent, by parental agents and displayed a preference for the L-isomer [Dantzig *et al.*, 1992]. Cephalexin, cefaclor and loracarbef competitively inhibited the initial uptake rates of both D-[9-^{14}C]cephalexin and D-[3-*phenyl*-^{3}H]cefaclor [Dantzig *et al.*, 1992]. These results suggest that a single H^+-dependent dipeptide transport carrier mediates the uptake of cephalexin and cefaclor in Caco-2 cells.

Cephradine accumulation in Caco-2 cells was temperature-dependent and sensitive to the pH of the apical compartment, suggesting that uptake is mediated *via* the DTS [Inui *et al.*, 1992]. Efflux from the basolateral membrane was also temperature-dependent, suggesting an active process. Uptake, which had a K_m of 8.3 mM and a V_{max} of 2.49 $nmol^{-1}$ mg $protein^{-1}$ min^{-1}, and the transport of cephradine were inhibitable by dipeptides, confirming that transport is *via* the DTS [Inui *et al.*, 1992].

Ceftibuten, is a cephalosporin which lacks an α-amino group, but has two carboxyl groups. Ceftibuten uptake was found to be pH-dependent and inhibited by dipeptides in Caco-2 cells, indicating that uptake is mediated *via* the DTS [Matsumoto *et al.*, 1994, 1995]. Four cephalosporins were found to be accumulated in Caco-2 cells in the following order, ceftibuten > cephradine > cephalexin > cefixime [Matsumoto *et al.*, 1994]. However, ceftibuten and cephradine both appeared in the basolateral compartment at the same rate [Matsumoto *et al.*, 1994]. Ceftibuten has a higher affinity for the apical DTS, but a lower affinity for the basolateral DTS when compared to cephradine, thus explaining the above results [Matsumoto *et al.*, 1994]. This also suggests that the basolateral DTS is the rate-limiting step for transcellular transport in Caco-2 cells [Matsumoto *et al.*, 1994].

Ceftibuten has a lower affinity ($K_m = 1.0$ mM) for the carrier but a higher capacity ($V_{max} = 0.40$ $nmol^{-1}$ mg $protein^{-1}$ min^{-1}), when compared to cephalexin [Matsumoto *et al.*, 1995]. The duration in culture had an effect on ceftibuten uptake and therefore on the development of the DTS [Matsumoto *et al.*, 1995]. Ceftibuten uptake in the presence of an H^+ gradient increased to d 14, after which it then decreased [Matsumoto *et al.*, 1995] Uptake in the absence of an H^+ gradient remained constant over the 14 d period studied. Kinetic analysis of the active component over the period studied indicated that ceftibuten uptake was affected by change in both the K_m and V_{max} values at d 14 and a decrease in V_{max} at d 21, resulting in a decline in uptake [Matsumoto *et al.*, 1995]. These results

suggest that cell growth and/or differentiation affect the development of the DTS in Caco-2 cells [Matsumoto *et al.*, 1995].

The oral β-lactam antibiotic, loracarbef, belongs to a new class called carbacephem, and is resistant to water and enzymatic hydrolysis compared to other β-lacatm antibiotics [Dantzig *et al.*, 1994a]. The uptake mechanisms of lorcarbef, which is nearly completely absorbed orally and cefixime, which is only 30-50 % absorbed, were compared in Caco-2 cells [Dantzig *et al.*, 1994a]. At intestinal pH values it will be a zwitterion, and therefore passive diffusion is unlikely. However, uptake of both compounds were *via* a carrier-mediated pathway (the DTS) and a diffusional process [Dantzig *et al.*, 1994a]. The kinetic parameters for lorcarbef uptake were K_m = 8.1 mM, V_{max} = 6.5 nmol^{-1} mg protein^{-1} min^{-1} and k_d = 0.19 nmol^{-1} mg protein^{-1} min^{-1} and for cefixime were K_m = 17 mM, V_{max} = 2 nmol^{-1} mg protein^{-1} min^{-1} and k_d = 0.17 nmol^{-1} mg protein^{-1} min^{-1} [Dantzig *et al.*, 1994a]. Lorcarbef has a similar affinity for the transporter as cephalexin [Dantzig & Bergin, 1990] and cefaclor [Dantzig *et al.*, 1992]. Cefixime and lorcarbef were competitive inhibitors of each others uptake, indicating that they share a common transport system [Dantzig *et al.*, 1994a]. Uptake of the prodrug cefuroxime axetil, and the parenterally administered cefuroxime, which both lack an α-amino group, were found to be *via* a passive process, with no involvement of the DTS [Dantzig *et al.*, 1994a]. Although the presence of an α-amino group is not essential for uptake*via* the DTS [Bai *et al.*, 1991], it may increase uptake by enhancing affinity for the transporter [Dantzig *et al.*, 1994a: Thwaites *et al.*, 1994a].

Further proof that the cephalosporins are absorbed by the DTS in Caco-2 cells has been provided [Thwaites *et al.*, 1994a]. Firstly, the orally absorbed cephalosporins cephradine, cefadroxil and cephalexin all inhibited the pH-stimulated transepithelial transport and intracellular accumulation of the dipeptide Gly-Sar [Thwaites *et al.*, 1994a]. Secondly, the inhibition profile is similar to that seen with cephradine [Inui *et al.*, 1992], and finally cephalexin and cefadroxil induced a marked intracellular acidification when perfused at the apical surface, consistent with dipeptide/H^+ cotransport [Thwaites *et al.*, 1994a]. Interestingly, the parenterally absorbed cefazolin did not significantly inhibit Gly-Sar accumulation, however, transport was inhibited by approximately 40 % [Thwaites *et al.*, 1994a]. Cefazolin produced a rapid initial acidification of the intracellular environment, which reached a plateau after 20 sec [Thwaites *et al.*, 1994a]. However, the response was significantly lower than that observed with cephalexin [Thwaites *et al.*, 1994a]. This suggests that either cefazolin is not a substrate for the apical DTS or that it has a lower affinity, thus explaining the poor oral absorption of this compound [Bergan, 1984], and that it has a higher affinity for the basolateral transporter [Thwaites *et al.*, 1994a].

Two apical and basolateral peptide transcellular transport pathways have been suggested to exist in Caco-2 cells [Hu *et al.*, 1994b]. Transcellular transport of lorcarbef across Caco-2 cell monolayers was found to involve an H^+ gradient-dependent process and a H^+ gradient-independent pathway [Hu *et al.*, 1994b]. The rate of the H^+ gradient-independent process was about one-quarter to one-half of that seen with the H^+ gradient-

dependent process [Hu *et al.*, 1994b]. The inhibition profiles for the two systems were different, dipeptides and peptidtyl-drugs inhibited the H^+ gradient-dependent process, whereas cephradine and enalapril were unable to inhibit lorcarbef transcellular transport in the absence of an H^+-gradient [Hu *et al.*, 1994b]. Also the inhibition with the dipeptides was lower in the absence of the H^+-gradient [Hu *et al.*, 1994b]. Other evidence for the presence of two pathways is the fact that the H^+ gradient-dependent process had a higher affinity and a lower capacity than the H^+ gradient-independent process. Also, in the presence of an H^+ gradient, Caco-2 cells were capable of concentrating lorcarbef, whereas in absence of the H^+ gradient they were unable to do so, suggesting that uptake is an H^+ gradient-dependent process [Hu *et al.*, 1994b]. Basolateral efflux of lorcarbef was faster in the absence of an H^+ gradient, suggesting efflux is mediated *via* a nonsaturable process [Hu *et al.*, 1994b]. Lorcarbef basolateral efflux was shown to have a carrier-mediated component [Hu *et al.*, 1994b], agreeing with a previous study that found the basolateral efflux of cephradine to be *via* a specific transport system [Inui *et al.*, 1992]. Hu *et al.* have proposed that lorcarbef apical uptake is *via* a carrier-mediated H^+ gradient-dependent process, whereas basolateral efflux is *via* a partially carrier-mediated H^+ gradient-independent process, which may not necessarily require energy [Hu *et al.*, 1994b].

Lorcarbef and cephalexin, which have been reported to have similar affinities for the peptide transporter in Caco-2 cells, K_m of 8.1 mM [Dantzig *et al.*, 1994a] and 7.5 ± 2.8 mM [Dantzig & Bergin, 1990] respectively, have been shown to have different affinities for a second transport component [Hu *et al.*, 1996]. Apical uptake was found to have higher K_m values and lower V_{max} values in the absence of an H^+ gradient, suggesting that the second transport component (H^+ gradient-independent process) is less significant than the first process in the presence of an H^+ gradient [Hu *et al.*, 1996]. Both lorcarbef and cephalexin where inhibited to comparable extents by a series of dipeptides and peptidyl-drugs, however as previously shown [Hu *et al.*, 1994], only dipeptides were capable of an inhibitory effect in the absence of an H^+ gradient [Hu *et al.*, 1996]. The protein kinase C promoter, PMA, which has been shown to down-regulate peptide transporter expression in Caco-2 cells [Brandsch *et al.*, 1994] decreased lorcarbef uptake in the presence of an H^+ gradient, but was without effect in the absence of an H^+ gradient [Hu *et al.*, 1996]. In the absence of an H^+ gradient feeding restriction affected lorcarbef uptake, whereas in the presence of an H^+ gradient uptake was unaffected [Hu *et al.*, 1996]. These results suggest that protein kinase C regulates a different component of transport compared to the one regulated by the feeding restriction, thus indicating evidence for the existence of two distinct processes responsible for lorcarbef uptake [Hu *et al.*, 1996]. There may be two active binding sites on the apical transporter or possibly two apical transporters, further studies are needed to evaluate the situation. However, the presence of two binding sites or transporters may explain the fact that cephalexin or cephradine were unable to inhibit SQ-29852 uptake in Caco-2 cells [Nicklin *et al.*, 1996].

6.2 MATERIALS AND METHODS

6.2.1 MATERIALS

Details of the suppliers of all materials used throughout this chapter have been described previously (section 2.1-2.1.3).

6.2.2 METHODS

6.2.2.1 MEDIA

Details of growth media (M1), and incubation media (M4) used throughout this chapter have been previously described (section 2.2.1.1).

6.2.2.2 CELL CULTURE

Cell line-1 (section 2.1.1) was used between passage numbers 25 to 38. The cells were seeded onto 24-well plates (2 cm^2) at a density of 1.6 x 10^5 cells $well^{-1}$, and grown in an atmosphere of 5 % CO_2 (95 % air) and 90 % relative humidity, at 37°C, as previously described (section 2.2.1.3). M1 was renewed every 48 h and the monolayers were used after 7-9 d growth, unless otherwise stated.

6.2.2.3 EXPERIMENTAL CONDITIONS FOR UPTAKE STUDIES

The following procedure and conditions were used for all studies unless otherwise stated: All solutions were preincubated at 37°C prior to experimentation, with the exception of PBS-azide which was kept at 4°C. The maintenance media were aspirated and the monolayers were washed with M4 at pH 6 (1 x 500 µl x 5 min) at 37°C. The incubation solution consisted of M4 with 2 % DMSO at pH 6, containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro, and the required amount of competitor (0.05, 0.1, 0.5, 1, 5 and 10 mM). Monolayers were incubated (1 x 250 µl x 3 min), at 37°C. Apical solutions were then collected, and the plates transferred to a cold table (4°C) and washed with ice-cold PBS-azide solution (2 x 500 µl x 5 min). The monolayers were then harvested by solubilising with 1 ml of 1 % v/v TX-100 in double distilled water. Apical solutions, washings and solubilised cells were collected, made up to 1 ml with double distilled water, added to 10 ml of HiSafe 3 or 15 ml Ecolite$^+$, depending on location, and counted for ^{3}H content by LSC. Results are corrected for 15 % non-specific uptake, and expressed % inhibition from the control value (Gly-[^{3}H]L-Pro uptake in the absence of competitor) and IC_{50} (mM) (Chapter 3, Equation 3.4). Data are mean values (of at least three monolayers) ± S.D. Competitors which produced inhibition values lower than 15 % or higher than 85 % were retested at a higher or lower concentration respectively, in order to achieve values within this range. Retesting of selected compounds was restricted due to availability of the compound in question and solubility problems. On occasions where the compounds were tested at more

than one concentration, all results are shown, however, solubility and material availability prevented retesting at the required concentrations of some compounds.

6.2.3 PEPTIDOMIMETIC DRUG INTERACTION WITH THE DTS

6.2.3.1 ACE-INHIBITOR INTERACTION WITH THE DTS

Uptake of Gly-[^{3}H]L-Pro in the presence of the following ACE-inhibitors were investigated at the concentration shown (Table 6.1); Fosinopril (0.1 mM), 4-cyclohexyl captopril (1 mM), SQ-29852 (1 mM), quinapril (1 mM), fosinoprilat (1 mM), benazepril (1 mM), cilazapril (1 mM), *S*-benzoyl captopril (1 mM), benazeprilat (5 mM), perindopril (5 mM), enalapril (10 mM), captopril (10 mM), enalaprilat (10 mM) and lisinopril (10 mM).

6.2.3.2 ACE-NEP INHIBITOR INTERACTION WITH THE DTS

Uptake of Gly-[^{3}H]L-Pro was measured in the presence of a range (43) of ACE-NEP inhibitors, which were divided into groups based upon structural variations where possible (Table 6.2 - 6.13). Molecular modelling was carried out on selected compounds using the Chem Pro 3D (CambridgeSoft) and Nemesis software (Oxford Molecular). Molecules were constructed and manipulated on screen to achieve various hypothetical conformations, with an energy minimisation then being performed.

6.2.3.3 β-LACTAM ANTIBIOTIC INTERACTION WITH THE DTS

Uptake of Gly-[^{3}H]L-Pro in the presence of the following β-lactam antibiotics were investigated at a 10 mM concentration (Table 6.14); Ampicillin, amoxicillin, cefaclor, cefazolin, cephalexin and cephradine.

6.3 RESULTS AND DISCUSSION

6.3.1 ACE-INHIBITOR INTERACTION WITH THE DTS

There has been some controversy regarding the absorption mechanisms of ACE-inhibitors across the GI tract. However, all of the ACE-inhibitors tested produced some degree of inhibition (Table 6.1). Fosinopril, which contains an esterified phosphinic acid group, had the greatest affinity for the transporter, $IC_{50} = 0.12 \pm 0.005$ mM. This was an unexpected result, as transport *in vivo* is believed not to involve the DTS, and reports in the literature state that the absorption in rats (single-pass perfusion method) is by a passive mechanism, without the involvement of the DTS [Friedman & Amidon, 1989b]. However, in *in vivo* studies, if active transport is present, it may be swamped by the passive component.

SQ-29852, which has been shown to be a stable and specific probe for the DTS [Marino *et al.*, 1996: Nicklin *et al.*, 1996], produced considerable inhibition of the probe as

expected due to the involvement of the DTS in its absorption (Table 6.1). Quinapril, fosinoprilat and benazepril, all produced IC_{50} values which were not significantly different from one another, 0.94 ± 0.23 mM, 1.03 ± 0.04 mM and 1.07 ± 0.09 mM respectively (Table 6.1). Quinapril transport has been shown to be *via* the DTS in Caco-2 cells, with a contribution from a non-saturable pathway [Hu *et al.*, 1995]. The present results indicate that quinapril does interact with the DTS, as shown by the displacement of the probe, and thus agree with the previous study [Hu *et al.*, 1995]. Fosinoprilat, which is the active diacid drug, and has been predicted to have < 5 % oral absorption in humans [Ranadive *et al.*, 1992], produced an IC_{50} of 1.03 ± 0.04 mM, which is 10-fold lower than that seen with the prodrug, fosinopril. Prodrugs have been designed to increase absorption, expected by virtue of higher distribution coefficients than that of their respective active moieties. In this case, values are 0.33 compared to 500 for fosinoprilat and fosinopril at pH 7 respectively, thus increasing passive absorption. The higher distribution coefficients seen with prodrugs may well have an effect on interaction with the DTS, thus explaining the difference in affinities. Prodrugs lose a negative charge, thus affecting the charge distribution of the molecule, which may have an impact on interaction with the DTS.

The esterified prodrug benazepril (Table 6.1) showed greater affinity for the DTS compared to its free diacid non-prodrug benazeprilat, IC_{50} values of 1.07 ± 0.09 mM and 5.02 ± 1.45 mM respectively. Benazepril uptake by rat everted intestinal rings was found to include a carrier-mediated component whereas uptake of benazeprilat was found to be passive [Kim *et al.*, 1994]. Although the results are in agreement with an active component mediating benazepril uptake, the fact that benazeprilat produced inhibition of the probe may suggest a difference in the DTS of Caco-2 cells and rat intestinal epithelial cells, or that it may simply be blocking the active site on the transporter.

Cilazapril, which has a bicyclic (seven and six membered ring) fused ring system in place of the proline ring, showed a high level of affinity for the transporter (IC_{50} = 1.34 ± 0.29 mM) (Table 6.1). The prodrug perindopril produced an IC_{50} of 5.66 ± 1.34 mM (Table 6.1), however, the active moiety perindopril, was without effect at 1 mM (data not shown). Unfortunately, the supply of perindopril was limited, and thus, retesting was restricted, preventing a comparison from being made.

Enalapril and enalaprilat (Table 6.1) produced similar degrees of activity, with IC_{50} values of 20.11 ± 2.04 mM and 30.21 ± 7.09 mM, respectively, which are not quite significantly different from one another at the 95 % confidence limits tested. The literature regarding enalapril absorption is somewhat controversial (see section 1.7.1). However, in Caco-2 cells, enalapril has been shown to cause intracellular acidification, providing direct evidence for H^+ coupled transport into the cell [Thwaites *et al.*, 1995] Our results are in agreement with this and suggest that enalapril is absorbed *via* the DTS. However, enalaprilat showed the same activity as enalapril, which is unusual, seeing that all of the other prodrugs (fosinopril and benazepril) tested have illustrated enhanced affinity for the transporter compared to the active moieties. Enalaprilat has been shown to inhibit

cephradine uptake into rabbit BBMV [Yuasa *et al.*, 1994], yet in rat small intestinal tissue, transport was found to be *via* a passive diffusional process [Swaan *et al.*, 1995]. The present data taken with previous literature, suggest that enalapril is absorbed *via* the DTS, in Caco-2, and that enalaprilat, although not a true substrate for the DTS causes inhibition of the probe by blocking the transporter site, without actually being translocated into the cell.

Captopril showed a low affinity for the DTS, with an IC_{50} of 24.20 ± 3.16 mM (Table 6.1). The absorption mechansims of captopril are somewhat controversial, although there is evidence for active transport *via* the DTS in Caco-2 cells [Thwaites *et al.*, 1995]. The present results suggest that captopril is a substrate for the DTS, although showing low affinity. Interestingly, the 4-cyclohexyl and *S*-benzoyl analogues of captopril both show considerably higher affinities for the transporter than the parent compound, IC_{50}s of 0.66 ± 0.04 mM and 1.43 ± 0.33 mM, respectively. This is in agreement with previous results (Chapter 4, section 4.3.1/3), illustrating that lipophilic functional groups in domains A and D increase interaction with the DTS.

The L-Lys-L-Pro analogue, lisinopril produced an of IC_{50} 32.25 ± 7.17 mM, displaying a weak affinity for the transporter (Table 6.1). The absorption mechansims of lisinopril are conflicting; results in Caco-2 cells have shown that it is a passive process, as it failed to induce cytosolic acidification when prefused at the apical surface [Thwaites *et al.*, 1995]. It has also been suggested that lisinopril is capable of binding to the DTS without undergoing translocation into the cell [Thwaites *et al.*, 1995]. However, lisinopril absorption in rat intestines has been shown to be absorbed *via* a non-passive mechanism [Friedman & Amidon, 1989a: Swaan *et al.*, 1995]. Our results are in agreement with the previous Caco-2 study [Thwaites *et al.*, 1995], and conclude that lisinopril is blocking the active site on the DTS, however, further investigation in animal models is required in order to provide conclusive evidence. Swaan *et al.*, demonstrated that lisinopril had an active transport process in rat intestine, and had a reduction in affinity for the transporter when compared to enalapril and enalaprilat due to intramolecular hydrogen bond formation [Swaan *et al.*, 1995]. However, the present study showed similar values for all three compounds.

TABLE 6.1 *The effect of a series of ACE-inhibitors on the uptake of Gly-[^{3}H]L-Pro*

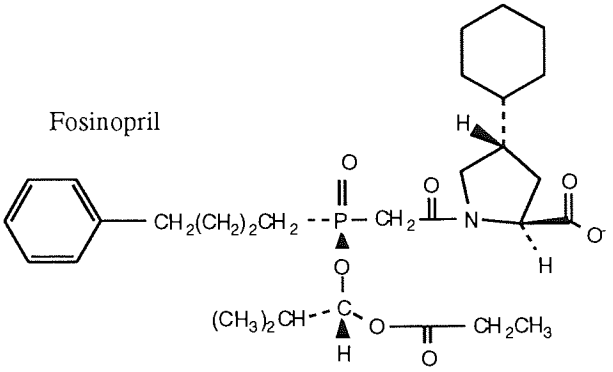
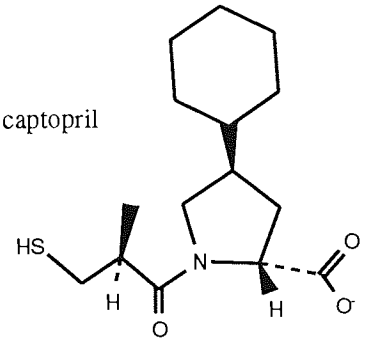
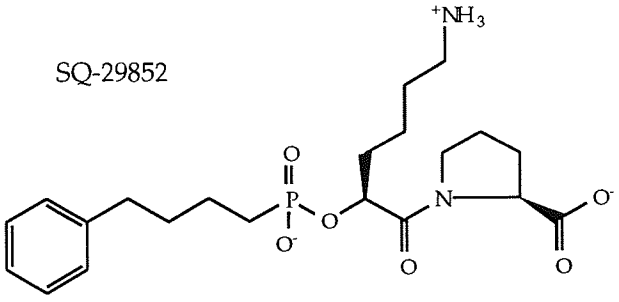
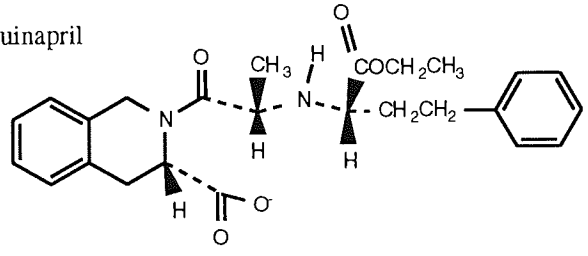
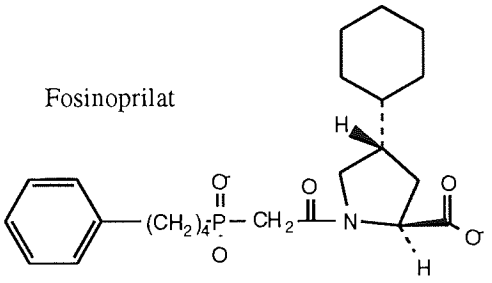
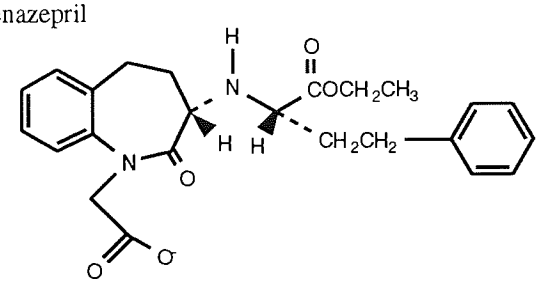
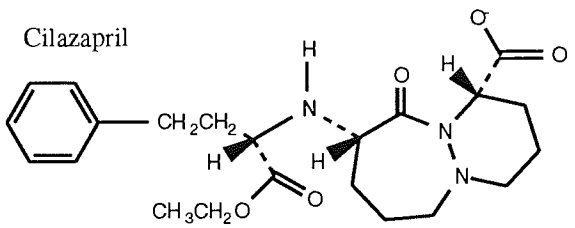
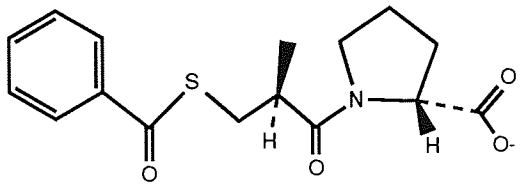
Competitor	Conc (mM)	% Inhibition Mean [S.D.]	IC_{50} (mM) Mean [S.D.]
Fosinopril 	0.5	97.56 [1.27][a]	0.013[0.007]
	0.1	47.28 [1.11][a]	0.11 [0.01]
4-cyclohexyl captopril 	1	60.28 [1.53][a]	0.66[0.04]
SQ-29852 	1	60.51[6.73][b]	0.68 [0.17]
Quinapril 	1	52.03[5.70][b]	0.94 [0.23]

TABLE 6.1 *The effect of a series of ACE-inhibitors on the uptake of Gly-[^{3}H]L-Pro, continued*

Fosinoprilat 	1	49.31[0.94][d]	1.03 [0.04]
Benazepril 	1	48.49 [2.15][a]	1.07 [0.09]
Cilazapril 	1	43.27[5.53][b]	1.34 [0.29]
S-benzoyl captopril 	1	41.58[5.20][b]	1.43 [0.33]

TABEL 6.1 *The effect of a series of ACE-inhibitors on the uptake of Gly-[^{3}H]L-Pro, continued*

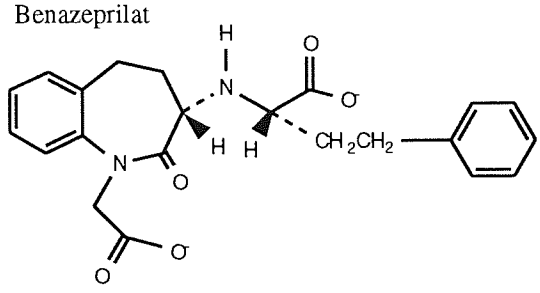
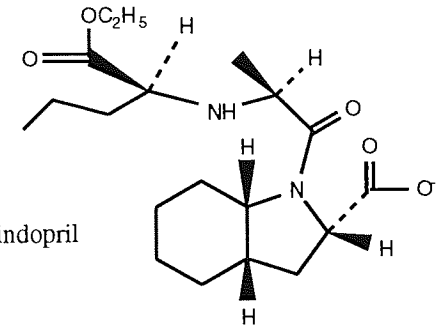
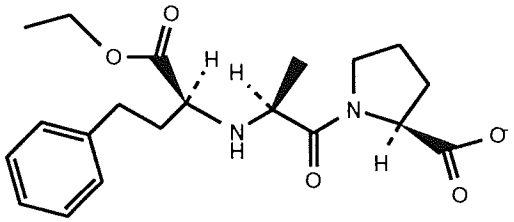
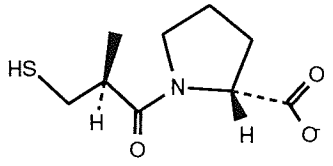
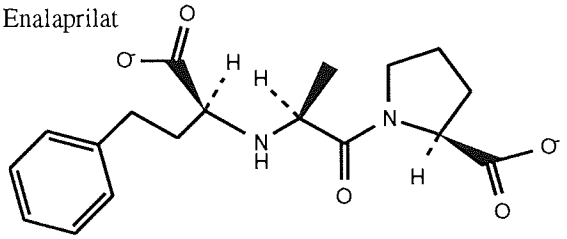
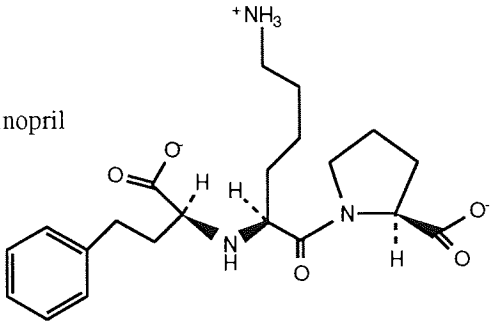
Benazeprilat		1	-13.68 [14.02]
		5	50.68 [7.96][c]
			y
			5.02 [1.45]
Perindopril		1	2.72 [7.64]
		5	47.43[6.07][b]
			y
			5.66 [1.34]
Enalapril		1	-11.6 [9.86]
		10	33.32[2.21][b]
			y
			20.11 [2.04]
Captopril		1	-8.73 [7.45]
		10	29.40 [2.59][c]
			y
			24.20 [3.16]

TABLE 6.1 *The effect of a series of ACE-inhibitors on the uptake of Gly-[^{3}H]L-Pro, continued*

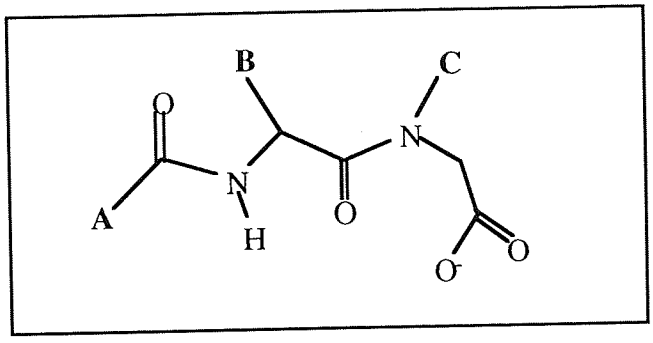
Enalaprilat 	1	-6.0 [8.30]	y
	10	25.43[4.76]^d	30.21 [7.09]
Lisinopril 	1	-7.33 [6.65]	y
	10	24.09[3.72]^d	32.25 [7.17]

Monolayers were washed (1 x 500 µl x 5 min) with M4. They were incubated with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6 in the presence of (0.1-10 mM) ACE-inhibitor. Data are corrected for 15 % non-specific uptake and presented as mean % inhibition ± S.D. for three monolayers and mean IC_{50} ± S.D., with ^{a}, ^{b}, ^{c} and ^{d} denoting significant difference at $p < 0.0001$, $p \leq 0.0005$, $p < 0.001$ and $p < 0.01$ respectively, in % inhibition from the control value (uptake in the absence of competitor). y corresponds to a % inhibition value which is too small (% inhibition ≤ 0.00) to be converted into a relevant IC_{50} value.

6.3.2 ACE-NEP INTERACTION WITH THE DTS

The ACE-NEP inhibitors are a class of drug that possess structural similarities to dipeptides. Most of the compounds tested have a carboxylate group and an amide function, as shown by the common fragment (Figure 6.1), with A, B and C corresponding to varied functional substituents examined in this work.

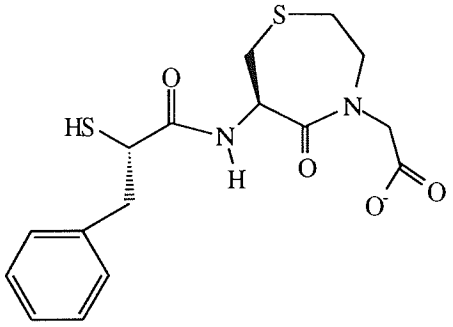
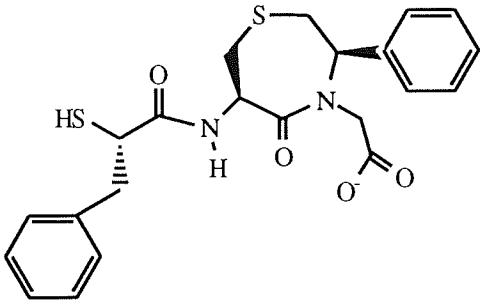
FIGURE 6.1 *Common fragment of the ACE-NEP structures*



Compounds BMS 184130 and BMS 184377 (Table 6.2) were used to explore the effect of increasing lipophilicity on interaction with the DTS. BMS 184130, at a 1 mM concentration had no effect on Gly-[^{3}H]L-Pro uptake, indicating either it is not a substrate for the DTS, or it has a low affinity and needs to be retested at a higher concentration to show an effect. However, when a phenyl group was substituted onto the seven-membered heterocyclic ring (BMS 184377), the affinity for the transporter was greatly increased (IC_{50} = 0.37 ± 0.06 mM), suggesting that lipophilicity in the region adjacent to the amide nitrogen at the *C*-terminus is important for interaction. This was also illustrated with the following series of compounds (Table 6.3). BMS 186309, which has a hydrophobic lipophilic cyclopentyl function at R, showed the greatest affinity for the DTS, with an IC_{50} of 0.08 ± 0.02 mM. The inhibition value at 92.70 ± 1.28 % falls outside the 85 % range, and therefore ideally the compound requires retesting at a lower concentration (*e.g.* 0.1 mM), although availability problems prevented this. However, the result clearly demonstrate that BMS 186309 displays the highest affinity for the transporter out of the four compounds in the series. BMS 186308 and BMS 187048, which both have C4 residues at R showed similar affinity for the transporter, IC_{50} of 0.19 ± 0.03 and 0.27 ± 0.03 mM respectively, although the results were significantly different at the confidence limits tested (95 %). BMS 188424, which has a hydrophilic hydroxyethyl substituent at R, displayed a much lower affinity for the transporter, with an IC_{50} value of 4.96 ± 0.85 mM. Lipophilic groups seem to increase interaction with the DTS, therefore the Log P values for the compounds were calculated using the Hansch principle [Hansch & Leo, 1979]. BMS 186309 had a calculated Log P contribution due to the cyclopentyl function of 3.0, whereas the Log P contribution of the hydroxyethyl substituent in BMS 188424 was -0.3. The Log P values for the C4 residues of BMS 186308 and BMS 187048 were very similar to one another, 2.45 and 2.7 respectively,

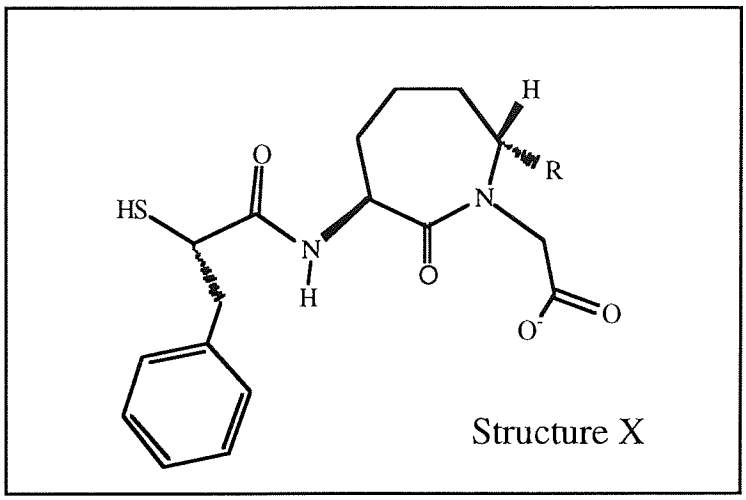
as were their IC_{50} values. Thus suggesting that increasing the lipophilicity at R will increase the interaction with the DTS. In a similar manner, bulky hydrophobic groups have also been shown to enhance the affinity of ACE-inhibitors for ACE [Pascard *et al.*, 1991].

TABLE 6.2 *ACE-NEP Inhibitors; The effect of increasing lipophilicity on interaction*

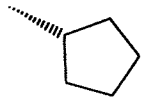
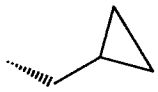
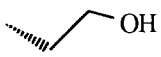
Compound	Conc (mM)	Structure	% Inhibition Mean [S.D.]	IC_{50} mM Mean [S.D.]
BMS 184130	1		2.24 [0.27]	y
BMS 184377	1		73.04 [3.12][a]	0.37 [0.06]

Monolayers were washed (1 x 500 µl x 5 min) with M4. They were then incubated with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6 in the presence of ACE-NEP inhibitors (for concentration see table). Data are corrected for 15 % non-specific uptake and presented as mean % inhibition ± S.D. for three monolayers and mean IC_{50} ± S.D., with [a] denoting a significant difference at $p < 0.0001$ in % inhibition from the control value (uptake in the absence of competitor). y corresponds to a % inhibition value which is too small (% inhibition ≤ 0.00) to be converted into a relevant IC_{50} value.

TABLE 6.3 ACE-NEP Inhibitors: *The effect of increasing lipophilicity at R*



Structure X

Compound	Conc (mM)	R group substituent	% Inhibition Mean [S.D.]	IC_{50} mM Mean [S.D.]
BMS 186309	1		92.70 [1.28][a]	0.08 [0.02]
BMS 186308	1		83.88 [1.73][a]	0.19 [0.03]
BMS 187048	0.1		26.81 [2.28][b]	0.27 [0.03]
BMS 188424	1		17.03 [2.59][c]	4.96 [0.85]

Monolayers were washed (1 x 500 µl x 5 min) with M4. They were incubated with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6 in the presence of ACE-NEP inhibitors (for concentration see table). Data are corrected for 15 % non-specific uptake and presented as mean % inhibition ± S.D. for three monolayers and mean IC_{50} ± S.D., with [a], [b] and [c] denoting significant difference at $p < 0.0001$, $p < 0.0005$ and $p < 0.005$, respectively, in % inhibition from the control (uptake in the absence of competitor).

BMS 186213 (Table 6.4) was a non-recognised compound at the concentration tested (1 mM). However, addition of a phenyl ring fused to the seven-membered heterocyclic ring and substituting a carbon in the seven-membered heterocyclic ring with a sulphur (BMS 186683) converted it into a recognised compound, IC_{50} of 0.75 ± 0.08 mM. Substituting the sulphur in BMS 186683 for an oxygen, as in BMS 186191, results in no significant change in affinity for the transporter, indicating that substitutions of atoms in the seven-membered ring are well tolerated. This also suggests that the difference in affinity seen between BMS 186213 and BMS 186683 is a result of the fused phenyl ring rather than substituting the sulphur atom. BMS 186213, 186683 and 186191 all have an increase in the length of the carboxylate chain compared to the common fragment, yet affinity is still seen with two of the compounds, illustrating that the length of the chain in domain E (Figure 4.1) can tolerate variation and still retain recognition. BMS 189701 has an hydroxyl group added to the phenyl ring at the *N*-terminus end of the molecule, which results in a reduction in affinity for the transporter compared to BMS 186191. However, in BMS 189701 the length of the carboxylate chain is reduced, which may also have an effect on interaction. BMS 185485 has the thiol group substituted with a thio acetyl function, which results in a decrease in affinity when compared to BMS 186683 and 186191. This area of the molecule correspond to group X in the hypothetical peptide substrate which has been proposed for interaction with the peptide transporter [Kramer *et al.,* 1995]. Group X is recommended to be a function capable of accepting protons, such as thiol. The thio acetyl is a weaker proton acceptor than the thiol group, due to the presence of the carbonyl function (electron withdrawing). By substituting the thiol (pKa -10) with an acetyl, although increasing the lipophilicity, the hydrogen bond capacity is markedly reduced. However, the oxygen of the carbonyl function may act as a H^+-acceptor due to its electron withdrawing functions, therefore possibly form hydrogen bonds. However, the present results are in agreement with the hypothetical structure [Kramer *et al.*, 1995].

TABLE 6.4 *ACE-NEP Inhibitors (1)*

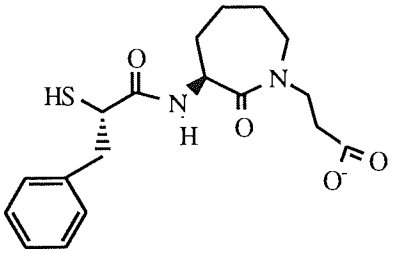
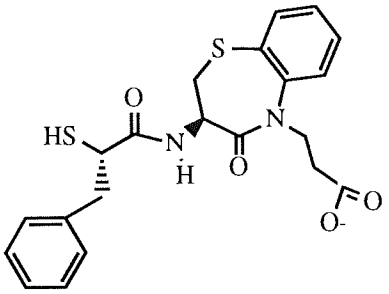
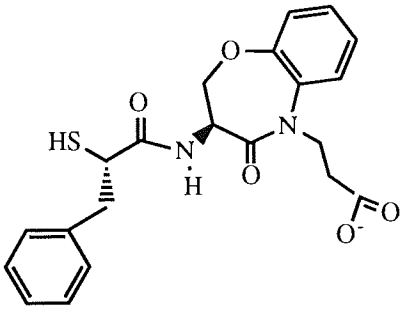
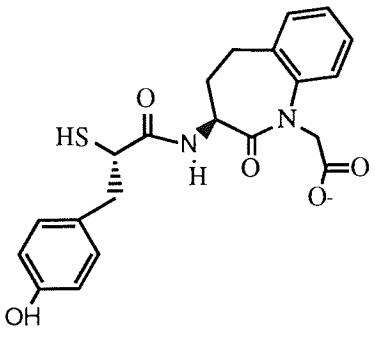
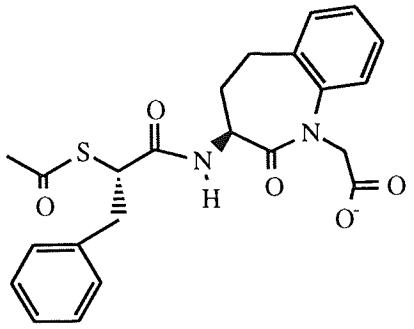
Compound	Conc (mM)	Structure	% Inhibition Mean [S.D.]	IC_{50} mM Mean [S.D.]
BMS 186213	1		-1.17 [20.50]	y
BMS 186683	1		57.32 [2.50][a]	0.75 [0.08]
BMS 186191	1		54.68 [0.77][a]	0.83 [0.03]
BMS 189701	1		39.84 [2.58][a]	1.22 [0.16]

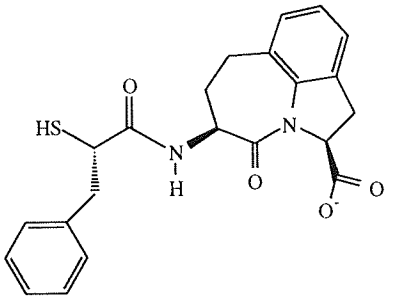
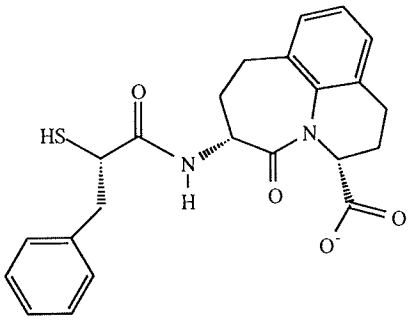
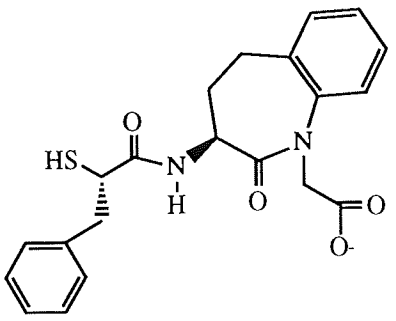
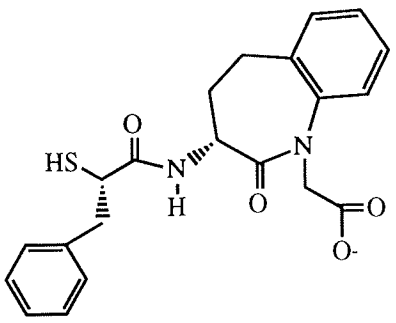
TABLE 6.4 *ACE-NEP Inhibitors (1), continued*

BMS 185485	1		27.29 [5.24]^{b}	2.77 [0.81]

Monolayers were washed (1 x 500 µl x 5 min) with M4. They were incubated with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6 in the presence of ACE-NEP inhibitors (for concentration see table). Data are corrected for 15 % non-specific uptake and presented as mean % inhibition ± S.D. for three monolayers and mean IC_{50} ± S.D., with ^{a} and ^{b} denoting significant difference at $p < 0.0001$ and $p < 0.005$, respectively, in % inhibition from the control (uptake in the absence of competitor). y corresponds to a % inhibition value which is too small (% inhibition ≤ 0.00) to be converted into a relevant IC_{50} value.

BMS 182943 (Table 6.5), which has the S-S stereochemistry at the *C*- and *N*-terminus displays a high affinity for the transporter, $IC_{50} = 0.07 \pm 0.01$. However, when the stereochemistry is altered to R-R, the compound (BMS 185838) loses all affinity, even when tested at a 10-fold higher concentration. For this series of peptidomimetic compounds, S-S stereochemistry corresponds to L-L dipeptides. This agrees with previous literature, stating that the DTS has a stereochemical preference for L-L compounds [Matthews, 1987: Thwaites *et al.*, 1994a]. BMS 184156 has the S (L) stereochemistry at the *N*-terminus, and displays no affinity for the transporter but, when the stereochemistry at the *N*-terminus is R (D) as in BMS 182657, the compound produced an IC_{50} of 1.38 ± 0.09 mM. This contrasts with previous results for BMS 182943 and BMS 185838. However, BMS 182657 and BMS 184156 differ from the common fragment as they have the carboxylate chain attached to the amide in the seven-membered heterocyclic ring, which has free rotation.

TABLE 6.5 *ACE -NEP Inhibitors: The effect of stereochemistry*

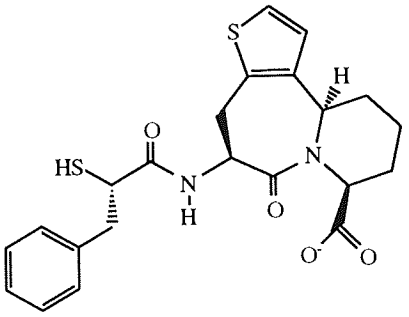
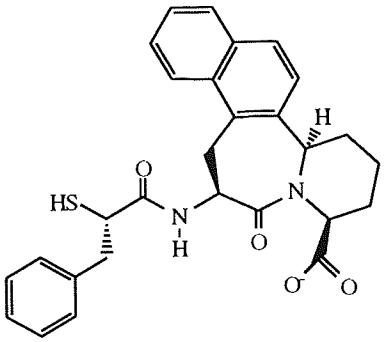
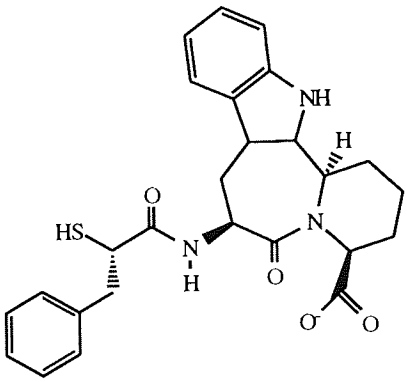
Compound	Conc mM	Structure	% Inhibition Mean [S.D.]	IC_{50} mM Mean[S.D.]
BMS 182943	1 0.1		94.63 [0.85]^a 58.93 [3.23]^a	0.057[0.01] 0.07 [0.01]
BMS 185838	1		-4.32 [7.37]	y
BMS 184156	1		3.23 [3.83]	y
BMS 182657	1		41.98 [1.56]^a	1.38 [0.09]

Monolayers were washed (1 x 500 µl x 5 min) with M4. They were incubated with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6 in the presence of ACE-NEP inhibitors (for concentration see table). Data are corrected for 15 % non-specific uptake and presented as mean % inhibition ± S.D. for three monolayers and mean IC_{50} ± S.D., with ^{a} denoting a significant difference at $p < 0.0001$ in % inhibition from the control (uptake in the absence of competitor). y corresponds to a % inhibition value which is too small (% inhibition ≤ 0.00) to be converted into a relevant IC_{50} value.

BMS 186047 (Table 6.6), which has a thiophene substituted on the lactam ring (the sulphur in the thiophene is a weak proton acceptor) inhibited all the active uptake of the probe at 1 mM (103.66 ± 1.01 % inhibition). Unfortunately, the unavailability of further compound prevented retesting at a lower concentration, preventing a relevant IC_{50} value from being obtained. The thiophene-substituted lactam ring might be considered analogous to L-Lys-L-Pro, which has been shown to be a good substrate for the DTS (Chapter 5, Table 5.2). Replacing the thiophene with a naphthalene function as in BMS 185201 produced a reduction in affinity compared to BMS 186047, which may possibly be due to steric effects. BMS 184708 has the naphthalene function replaced with an indole which resulted in a reduction in affinity. The inhibition value for BMS 185201 falls outside the desired range, and thus ideally needs retesting.

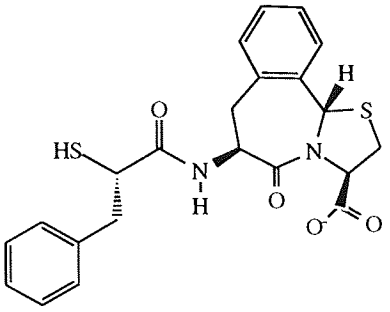
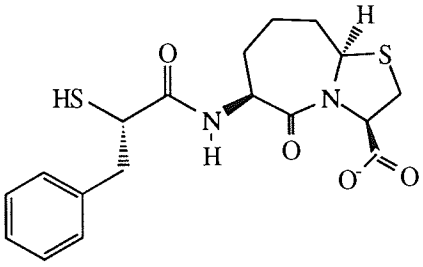
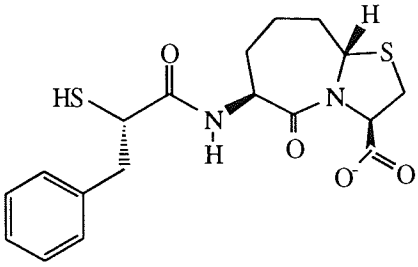
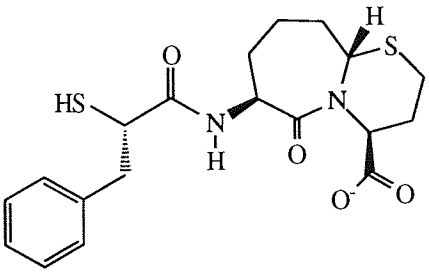
BMS 187080 (Table 6.7), which has a phenyl ring fused to the seven-membered heterocyclic ring shows good affinity for the DTS producing an IC_{50} of 0.22 ± 0.03 mM. By removing the phenyl ring, and thus decreasing the lipophilicity as in BMS 187375, activity is reduced, IC_{50} = 1.03 ± 0.08 mM. When the fused five-membered ring is replaced with a six-membered ring (BMS 187639) affinity is reduced considerably, IC_{50} = 2.89 mM; this may be due to a change in the length between the carbonyl of the carboxylate and the ring carbonyl. However, molecular modelling is required to confirm this theory. BMS 182907 shows an increase in recognition compared to BMS 187375, IC_{50} values of 0.44 ± 0.04 mM compared to 1.03 ± 0.08 mM respectively. This is a result of a change in the stereochemistry of the proton at the bridge position between the fused rings. In BMS 187080, 187375 and 187639 the proton at the bridge position between the fused rings is *cis* in respect to the carboxylate function, whereas in BMS 182907 the stereochemistry is changed to the *trans* configuration, and has the effect of increasing affinity for the DTS.

TABLE 6.6 *ACE-NEP Inhibitors (2)*

Compound	Conc mM	Structure	% Inhibition Mean [S.D.]	IC_{50} mM Mean[S.D]
BMS 186047	1		103.66 [1.01][a]	x
BMS 185201	0.05		13.94 [4.18][c]	0.33 [0.10]
BMS 184708	0.5		15.37 [2.85][b]	2.84 [0.68]

Monolayers were washed (1 x 500 µl x 5 min) with M4. They were incubated with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6 in the presence of ACE-NEP inhibitors (for concentration see table). Data are corrected for 15 % non-specific uptake and presented as mean % inhibition ± S.D. for three monolayers and mean IC_{50} ± S.D., with [a], [b] and [c] denoting significant difference at $p < 0.0001$, $p < 0.005$ and $p < 0.01$, respectively, in % inhibition from the control (uptake in the absence of competitor). x corresponds to a % inhibition value which is too large (% inhibition ≥ 0.00) to be converted into a relevant IC_{50} value.

TABLE 6.7 *ACE-NEP Inhibitors (3)*

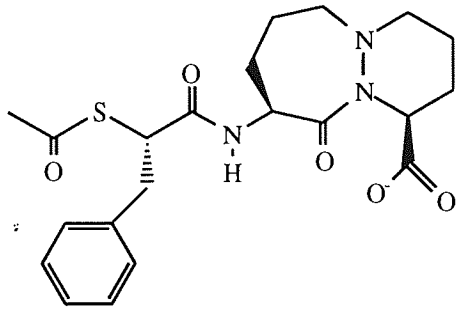
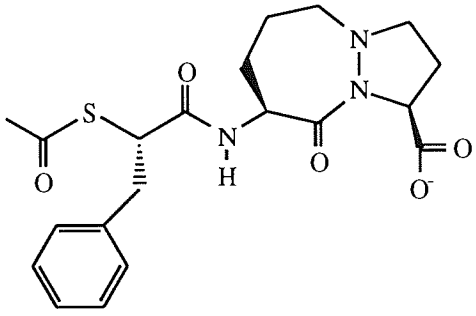
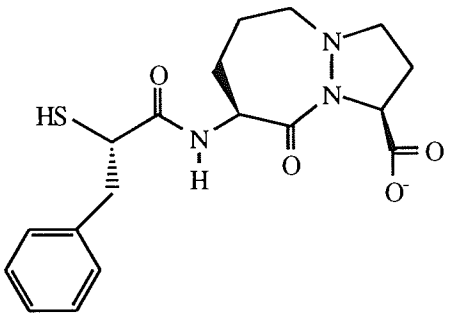
Compound	Conc mM	Structure	% Inhibition Mean [S.D.]	IC_{50} mM Mean [S.D.]
BMS 187080	0.5		69.30 [2.89][a]	0.22 [0.03]
BMS 182907	0.5		53.24 [2.26][a]	0.44 [0.04]
BMS 187375	1		49.39 [1.81][a]	1.03 [0.08]
BMS 187639	1		26.16 [4.36][b]	2.89 [0.62]

Monolayers were washed (1 x 500 µl x 5 min) with M4. They were incubated with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6 in the presence of ACE-NEP inhibitors (for concentration see table). Data are corrected for 15 % non-specific uptake and presented as mean % inhibition ± S.D. for three monolayers and mean IC_{50} ± S.D., with [a] and [b] denoting significant difference at $p < 0.0001$ and $p < 0.001$, respectively, in % inhibition from the control (uptake in the absence of competitor).

BMS 188383 (Table 6.8), which has a seven-membered heterocyclic ring fused to a six-membered ring, produced an IC_{50} value of 0.63 ± 0.12 mM. BMS 188267 has the six-membered ring substituted with a five-membered ring, which results in a decrease in affinity, producing an IC_{50} of 0.92 ± 0.03 mM. The contradicts with previous data (Table 6.7) which showed that substituting with a six-membered ring in BMS 187639, resulted in decreased affinity compared to BMS 187375, which possess a five-membered ring. BMS 188178 is BMS 188267 with a thiol in place of the acetyl substitution on the sulphur, shows a decrease in interaction with the DTS. These findings do not support previous interpretations where we have shown that acetylation of the thiol reduced affinity (BMS 185485) agreeing with hypothetical peptide structure [Kramer *et al.*, 1995]. This demonstrates that the functional group suggested by Kramer *et al.*, for this region, does not hold true for all structures, whereby the presence of multiple recognition sites on the molecule, may override the deleterious effect of any one change.

Molecular modelling of BMS 182907 (Figure 6.2), BMS 187375 (Figure 6.3), BMS 188383 (Figure 6.4) and BMS 188267 (Figure 6.5) were carried out. In all four structures, it is possible to orient the carbonyl functions of the carboxylate, ring amide and side-chain amide in a similar direction. This orientation can be largely maintained in an energy minimised state derived from these conformations. These may not be the true lowest energy conformations but represents a reasonable local minimum. As these structures favour the similar orientation of the carbonyls, this suggests that they may be important in forming hydrogen bonds with the transporter. Such hydrogen bonding is known to be involved in the interaction of ACE-inhibitors with the three hydrophobic pockets in ACE [Pascard *et al.*, 1991]. Factors which favour this type of orientation, such as stereochemistry, might improve recognition with the transporter.

TABLE 6.8 *ACE-NEP Inhibitors; Aza analogues*

Compound	Conc mM	Structure	% Inhibition Mean [S.D.]	IC_{50} mM Mean [S.D.]
BMS 188383	0.1		13.97 [2.39][b]	0.63 [0.12]
BMS 188267	1		52.11 [0.75][a]	0.92 [0.03]
BMS 188178	1		42.07 [2.06][a]	1.38 [0.12]

Monolayers were washed (1 x 500 µl x 5 min) with M4. They were incubated with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6 in the presence of ACE-NEP inhibitors (for concentration see table). Data are corrected for 15 % non-specific uptake and presented as mean % inhibition ± S.D. for three monolayers and mean IC_{50} ± S.D., with [a] and [b] denoting significant difference at $p < 0.0001$ and $p < 0.005$, respectively, in % inhibition from the control (uptake in the absence of competitor).

FIGURE 6.2 *BMS 182907*

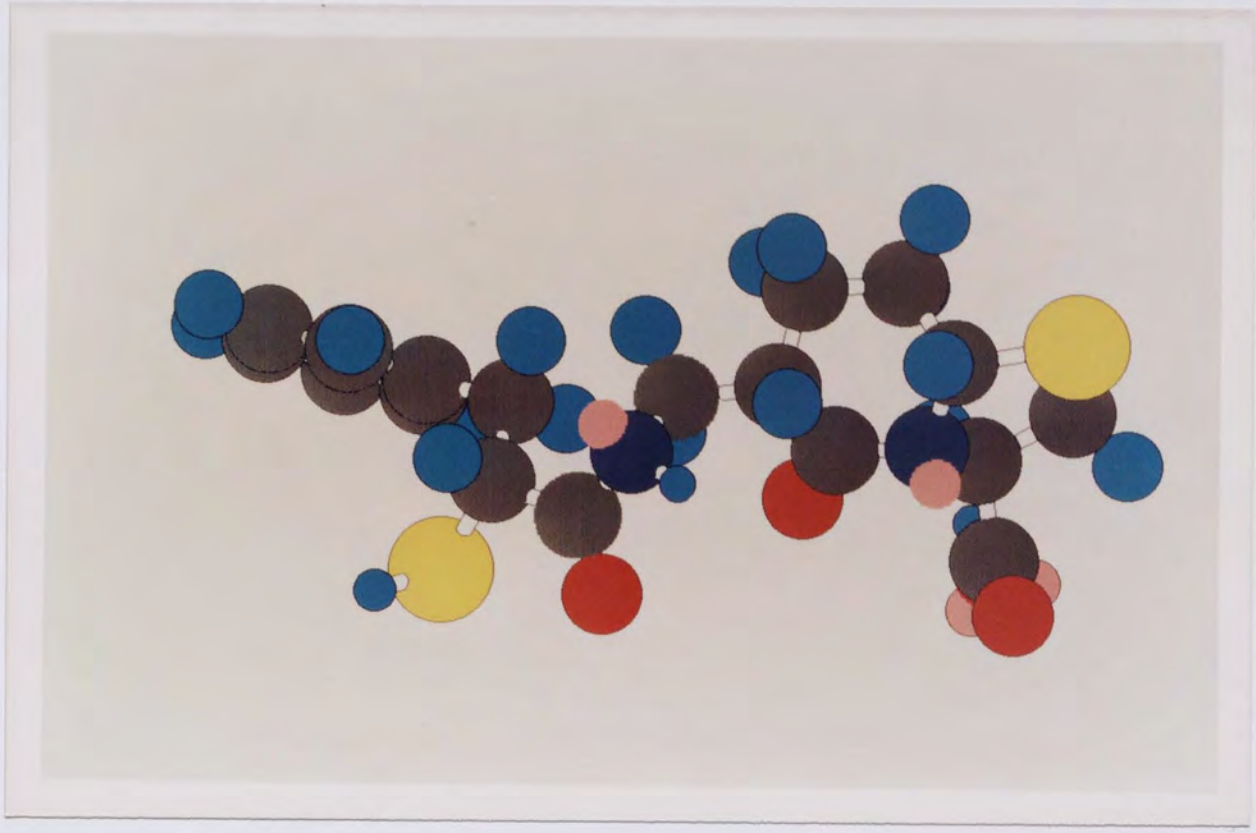


FIGURE 6.3 *BMS 187375*

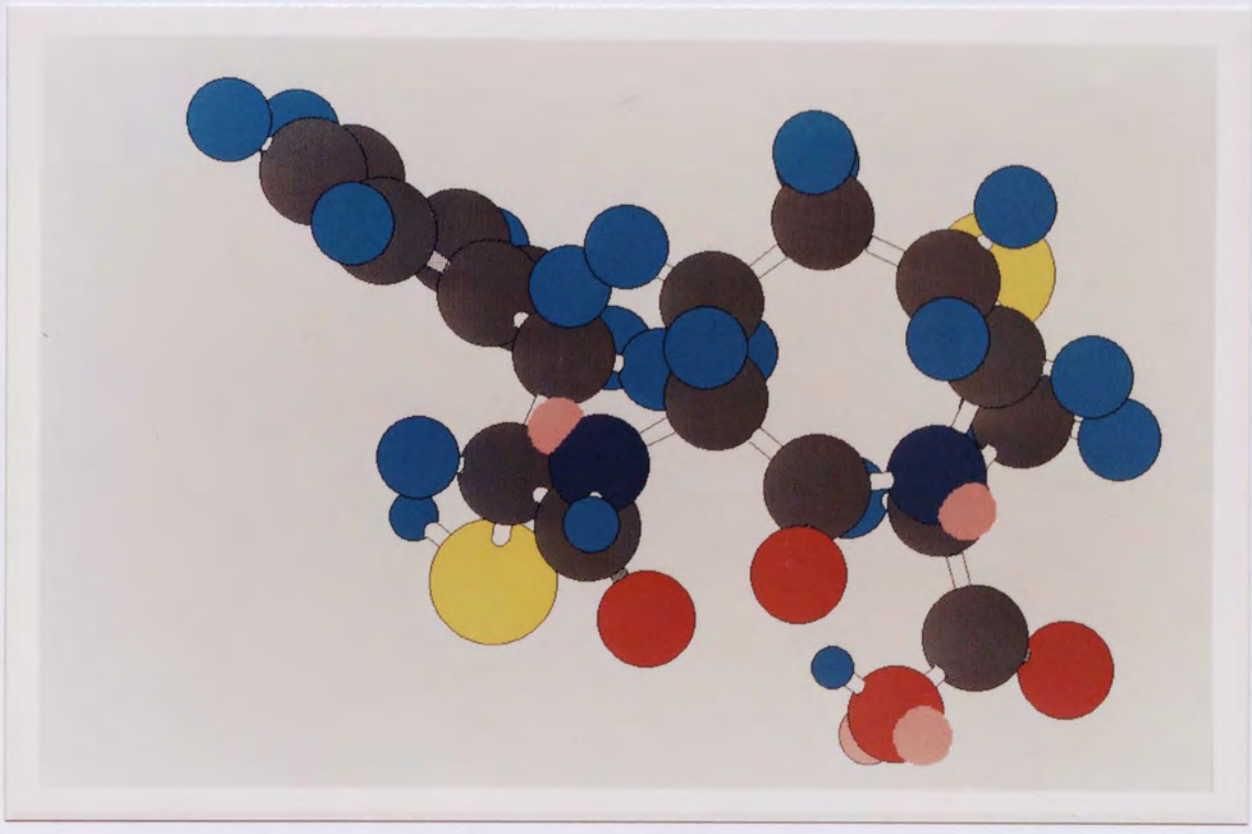


FIGURE 6.4 *BMS 188383*

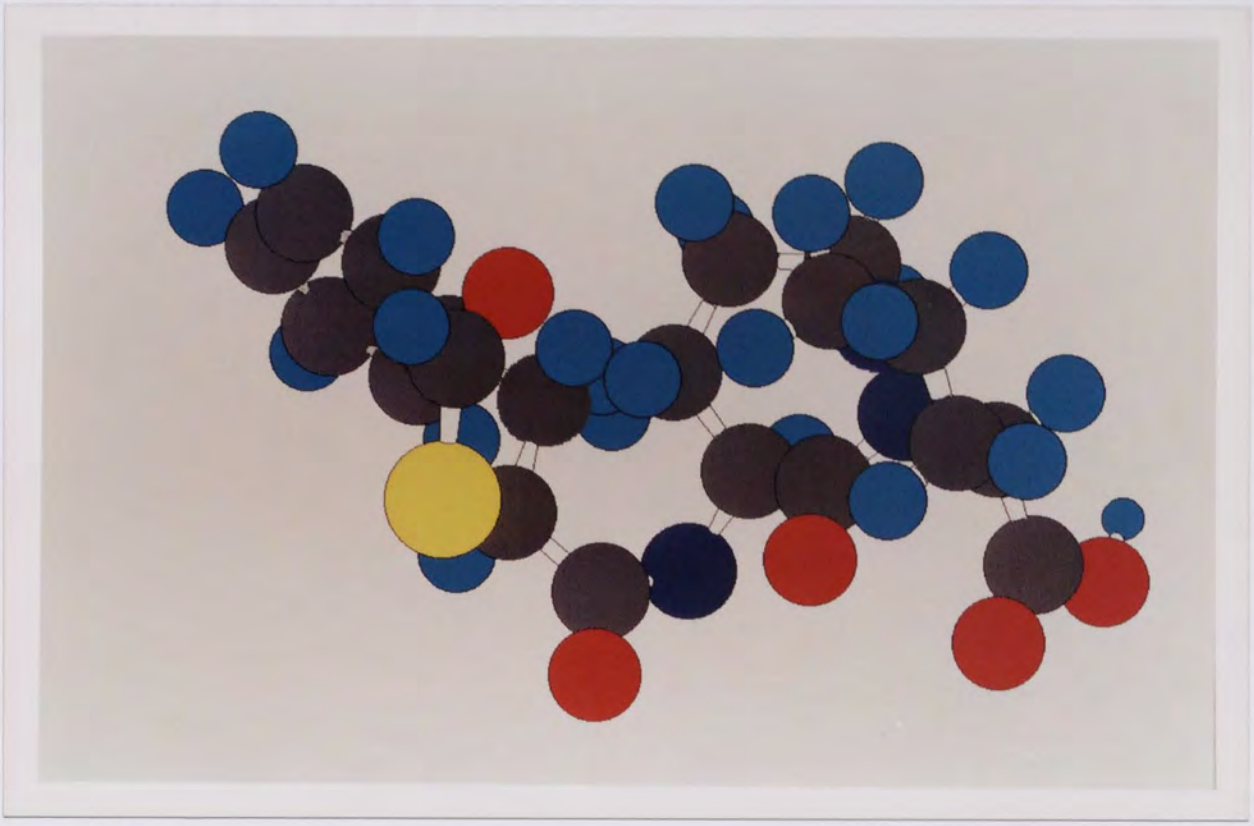


FIGURE 6.5 *BMS 188267*

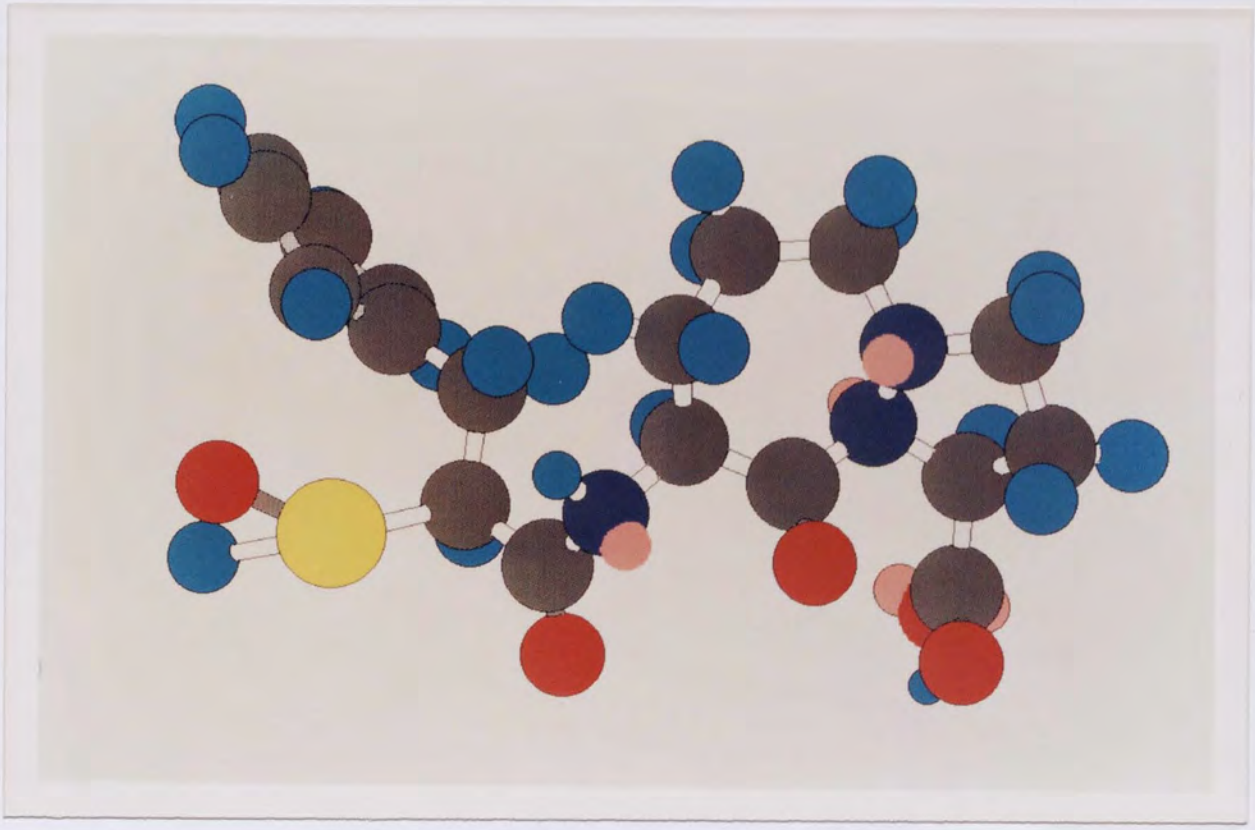
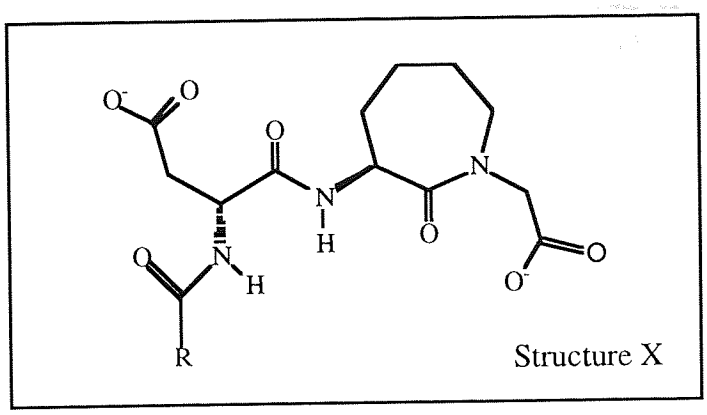


Table 6.9 shows a series of compounds in which substitutions are made to the *N*-terminal region of the molecule. BMS 193669 which has a phenyl ring at R displayed the highest affinity for the DTS, IC_{50} = 1.27 ± 0.30 mM. Replacing the phenyl ring with a benzyloxy function produced a reduction in affinity giving an IC_{50} of 6.62 ± 1.90 mM. When the phenyl ring was substituted with an ethyl function (BMS 194043) or a t-butoxy function (BMS 193670), affinity is abolished. These results suggest that a bulky lipophilic function at R increases affinity for the transporter. However, the inhibition values for BMS 193669 and BMS 193893 are not within the required range (15 - 85 %), and therefore, although an estimation of affinity is gained, the compounds need retesting at a higher concentration. BMS 194043 and BMS 193670 are non-inhibitors at 1 mM and therefore ideally should be retested at a higher concentration (10 mM).

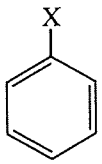
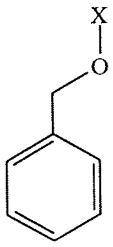
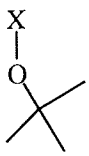
BMS 186718 (Table 6.10) and BMS 188094 again illustrate that a thiol function at group X is recommended for optimum interaction [Kramer *et al.*, 1995]. Replacing the thiol or the thio acetyl function at group X, with a carboxylate function (BMS 194077), thus making the molecule doubly anionic, and substituting a secondary amide function, results in a decrease in affinity. BMS 188035 has the six-membered fused ring in BMS 186718 substituted with a five-membered ring, resulting in a four-fold decrease in affinity.

BMS 185503 and BMS 186019 (Table 6.11) illustrate that the stereochemistry of the functional groups attached to the seven-membered heterocyclic ring, at a position believed to be distinct from the initial dipeptide recognition site, has an impact on overall interaction with the DTS. BMS 186194 is the same as BMS 186718, except that the sulphur in the seven-membered heterocyclic ring has been substituted with a oxygen, resulting in a decrease in affinity for the DTS (BMS 186718, IC_{50} = 0.09 ± 0.01 mM, compared to BMS 186194, IC_{50} = 1.53 ± 0.30 mM). The oxygen is slightly more hydrophilic than sulphur, which could explain the large decrease in affinity. However, it has been previously shown that the seven-membered heterocyclic ring is tolerant to atom substitutions (Table 6.4), and therefore, again illustrates that multiple recognition sites on the molecule may override the deleterious effect of any one change.

TABLE 6.9 *ACE-NEP Inhibitors (4)*

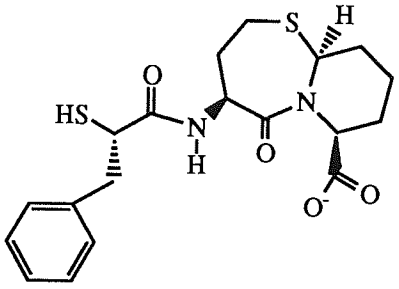
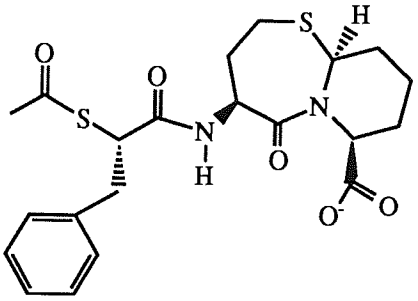
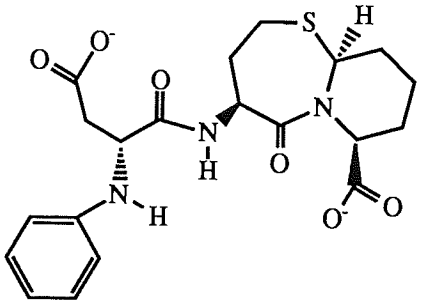
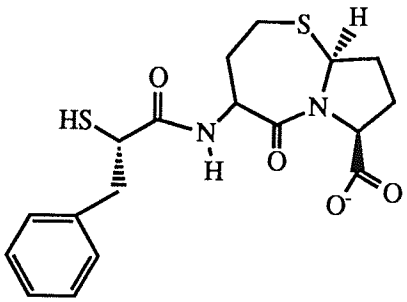


Structure X

Compound	Conc mM	R group substituent	% Inhibition Mean [S.D.]	IC_{50} mM Mean [S.D.]
BMS 193669	0.1		7.57 [1.86][b]	1.27 [0.30]
BMS 193893	1		13.61 [2.97][a]	6.62 [1.90]
BMS 194043	1		3.42 [10.25]	y
BMS 193670	1		7.76 [8.71]	y

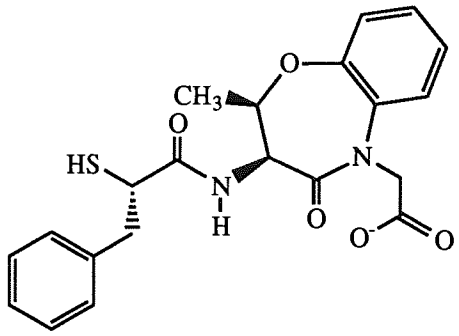
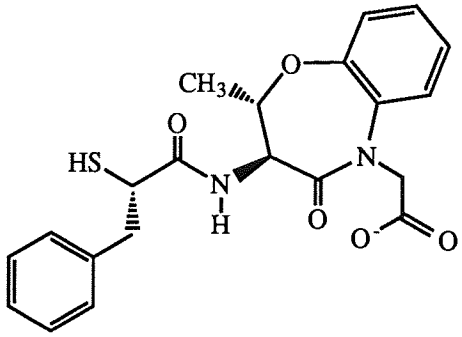
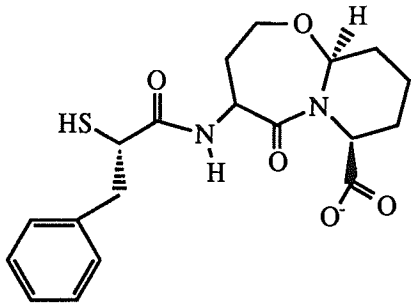
Monolayers were washed (1 x 500 µl x 5 min) with M4. They were incubated with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6 in the presence of ACE-NEP inhibitors (for concentration see table). Data are corrected for 15 % non-specific uptake and presented as mean % inhibition ± S.D. for three monolayers and mean IC_{50} ± S.D., with [a] and [b] denoting significant difference at $p < 0.005$ and $p < 0.05$, respectively, in % inhibition from the control (uptake in the absence of competitor). y corresponds to a % inhibition value which is too small (% inhibition ≤ 0.00) to be converted into a relevant IC_{50} value.

TABLE 6.10 *ACE-NEP Inhibitors (5)*

Compound	Conc mM	Structure	% Inhibition Mean [S.D.]	IC_{50} mM Mean [S.D.]
BMS 186718	0.1		53.45 [2.48][a]	0.09 [0.01]
BMS 188094	1		84.61 [0.45][b]	0.18 [0.01]
BMS 194077	1		32.84 [0.62][a]	2.05 [0.06]
BMS 188035	1		73.76 [2.17][a]	0.36 [0.04]

Monolayers were washed (1 x 500 µl x 5 min) with M4. They were incubated with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6 in the presence of ACE-NEP inhibitors (for concentration see table). Data are corrected for 15 % non-specific uptake and presented as mean % inhibition ± S.D. for three monolayers and mean IC_{50} ± S.D., with [a] and [b] denoting significant difference at $p < 0.0001$ and $p < 0.0005$, respectively, in % inhibition from the control (uptake in the absence of competitor).

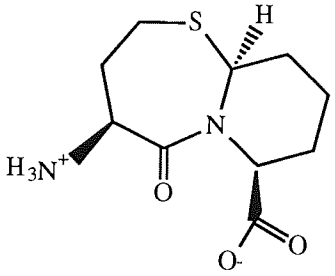
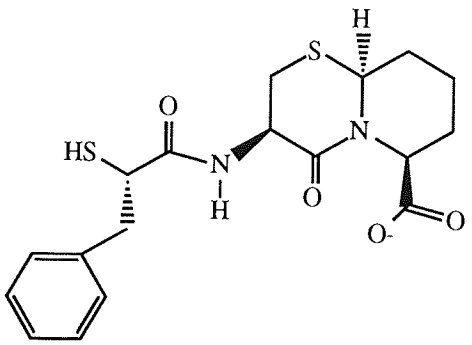
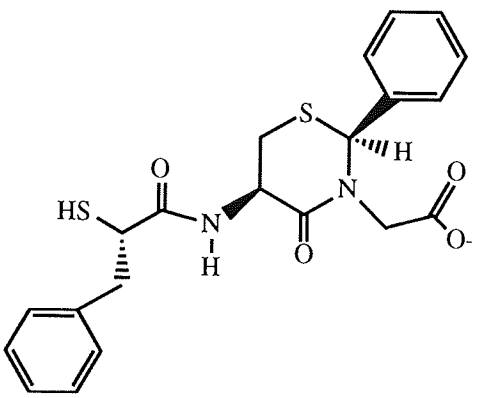
TABLE 6.11 *ACE-NEP Inhibitors (6)*

Compound	Conc mM	Structure	% Inhibition Mean [S.D.]	IC_{50} mM Mean [S.D.]
BMS 185503	1		42.84 [2.37][a]	1.34 [0.13]
BMS 186019	1		52.51 [1.06][a]	0.91 [0.04]
BMS 186194	1		39.80 [4.40][b]	1.53 [0.30]

Monolayers were washed (1 x 500 µl x 5 min) with M4. They were incubated with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6 in the presence of ACE-NEP inhibitors (for concentration see table). Data are corrected for 15 % non-specific uptake and presented as mean % inhibition ± S.D. for three monolayers and mean IC_{50} ± S.D., with [a] and [b] denoting significant difference at $p < 0.0001$ and $p < 0.0005$, respectively, in % inhibition from the control (uptake in the absence of competitor).

BMS 196099 (Table 6.12) which has a seven-membered sulphur containing heterocyclic ring fused to a six-membered ring, and possess a primary amine at the *N*-terminus still retains affinity for the transporter. BMS 186684 which has the seven-membered ring replaced by a six-membered ring is still compatible with the DTS.

TABLE 6.12 *ACE-NEP Inhibitors (7)*

Compound	Conc mM	Structure	% Inhibition Mean [S.D.]	IC_{50} mM Mean [S.D.]
BMS 196099	0.1		27.51 [1.67][a]	0.26 [0.02]
BMS 186684	1		61.92 [1.83][a]	0.62 [0.05]
BMS 183341	0.5		33.4 [2.55][a]	1.00 [0.12]

Monolayers were washed (1 x 500 µl x 5 min) with M4. They were incubated with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6 in the presence of ACE-NEP inhibitors (for concentration see table). Data are corrected for 15 % non-specific uptake and presented as mean % inhibition ± S.D. for three monolayers and mean IC_{50} ± S.D., with [a] denoting significant difference at $p \leq 0.0001$, in % inhibition from the control (uptake in the absence of competitor).

DER 37488-115-27 (Table 6.13) which has a seven-membered ring containing a carbonyl, an ethylcarboxylate function and an amine displayed no affinity for the DTS at the concentration tested. Fusing a phenyl ring between positions 2 and 3 of the seven-membered ring (DER 37488-115-25) converted it into an active compound, IC_{50} = 2.64 ± 1.26, illustrating that lipophilic functions are essential for recognition. BMS 183524, containing an eight-membered ring in place of the seven-membered ring, still retained affinity for the transporter. Interestingly, compounds possessing an eight-membered lactam ring have also been shown to bind to ACE [Watthey *et al.*, 1984]. SQ-028229, which lacks a ring structure at the *C*-terminus end of the molecule, produced an IC_{50} of 1.15 ± 0.10 mM, illustrating that a ring structure is not essential for recognition.

6.3.3 β-LACTAM INTERACTION WITH THE DTS

All the competitors tested, inhibited the probe to some extent (Table 6.14), but displayed lower affinities for the transporter than the natural dipeptides substrates, as seen with a previous study [Eddy *et al.*, 1995]. This may be due to the fact that part of the antibiotic structure resembles that of the unnatural dipeptide D-Ala-D-Ala, which is only present in nature in bacterial cell walls. The DTS has been shown to have a preference for dipeptides containing the L-enantiomers of amino acids rather than the D-enantiomers (Chapter 5) [Matthews, 1987: Thwaites *et al.*, 1994a: Hidalgo *et al.*, 1995]. The cephalosporins produced a greater extent of inhibition (68.1 ± 5.83 - 33.5 ± 6.90 %) compared to that seen with the penicillins. Previous studies using Caco-2 cell monolayers, have shown that cefaclor, cephradine and cephalexin are all substrates for the DTS showing similar affinities with K_m values of 7.6 mM, 7.5 mM and 8.3 mM respectively [Dantzig & Bergin, 1990: Dantzig *et al.*, 1992: Inui *et al.*, 1992]. In the present model, although the result for cefaclor, cephradine and cephalexin are significantly different from one another at the confidence limits tested (95 %), they are all within the same order of magnitude. Cefaclor, which is a close structural analogue of cephalexin, was the most active compound with an IC_{50} of 4.77 ± 1.33 mM. Cephradine showed slightly better affinity for the transporter than cephalexin (IC_{50} of 7.72 ± 0.69 mM compared to 9.55 ± 0.51 mM), and produced an IC_{50} value which was comparable with a previous K_i value of 4.50 ± 1.53 mM, shown in the Caco-2 cell model [Eddy *et al.*, 1995]. The parenterally administered cefazolin, which lacks a α-amino group had considerably lower affinity for the DTS compared with the previous cephalosporins, IC_{50} of 20.7 ± 5.64 mM, which would be consistent with poor oral absorption. Previously, cefazolin has been reported to have a reduced affinity for the DTS in Caco-2 cells compared to the orally administered cephalexin [Thwaites *et al.*, 1994a], which is consistent with the present study.

Surprisingly, the penicillins, ampicillin and amoxicillin produced IC_{50} values which were not significantly different from one another ($p > 0.05$). Amoxicillin has been reported to have a K_m of 0.06 ± 0.03 mM, which is several hundred times lower than that for ampicillin (K_m = 15.8 ± 2.92 mM), in a study using rat intestine [Oh *et al.*, 1992]; however,

this was not demonstrated in the present study. This suggests a much greater affinity for the DTS which seems surprising considering that the structure of amoxicillin only differs from that of ampicillin by the presence of a hydroxyl group on the benzene ring. However, this is an ionisable group, and therefore creates another site for interaction between the structure and the receptor site of the DTS. A previous study, has reported K_i values of 9.60 ± 0.63 and 5.79 ± 0.67 mM for amoxicillin and ampicillin respectively, which differ from the present values, however cephalexin was used as the probe compound and the Caco-2 cells were at a higher passage number, 65-80. Thus offering a possible explanation for the differences, however, interestingly the values for cephradine are similar between studies.

The carbapenems are a new class of β-lactam antibiotics, which differ from the α-amino β-lactam antibiotics by having a hydroxy group instead of the amino function at position 6 or 7 of the nucleus. The two carbapenems HR 664 and SUN 5555 failed to inhibit cephalexin uptake into rabbit BBMV, and HR 664 uptake was unaffected by DTS substrates, suggesting that the carbapenems are not substrates for the DTS [Kramer *et al.*, 1995]. All the carbapenems (imipenem, meropenem, panipenem and BO-2727) tested, with the exception of biapenem which produced 9.21 ± 0.70 % inhibition, failed to inhibit Gly-[^{3}H]L-Pro uptake into Caco-2 cells at a 1 mM concentration (data not shown). This indicates that the carbapenems are not substrates for the DTS as previously suggested [Kramer *et al.*, 1995], or that they have a low affinity for the transporter, and therefore, require retesting at a higher concentration.

TABLE 6.13 *ACE-NEP Inhibitors (8)*

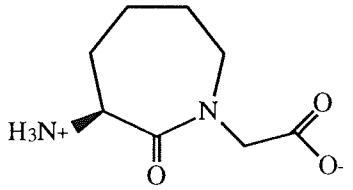
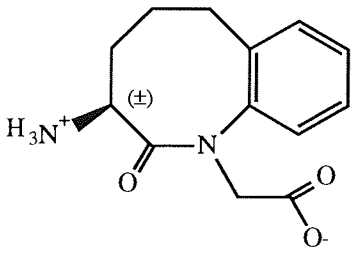
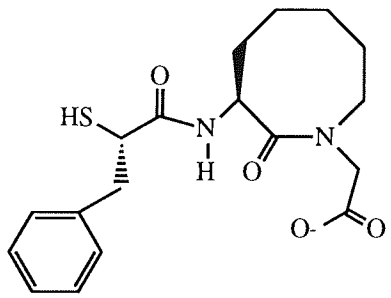
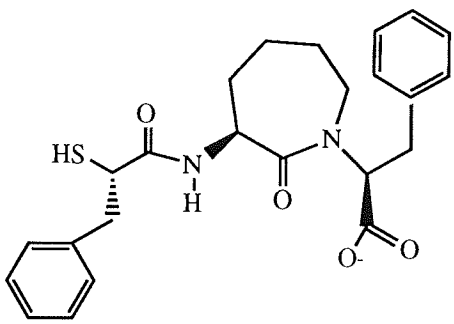
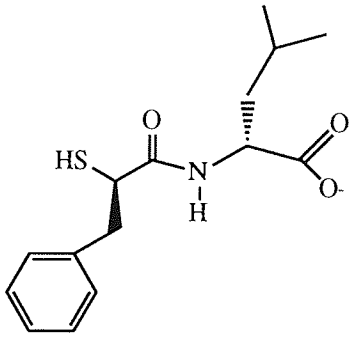
Compound	Conc mM	Structure	% Inhibition Mean [S.D.]	IC_{50} mM Mean [S.D.]
DER 37488-115-27	0.5		3.66 [4.33]	y
DER 37488-115-25	0.5		17.43 [5.70][c]	2.64 [1.26]
BMS 184534	1		26.48 [3.41][b]	2.82 [0.47]
BMS 185160	1		70.95 [1.83][a]	0.41 [0.04]

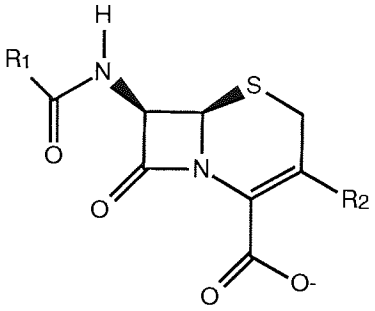
TABLE 6.13 *ACE-NEP Inhibitors (8)*

SQ-028229	0.5		30.38 [1.75][a]	1.15 [0.10]

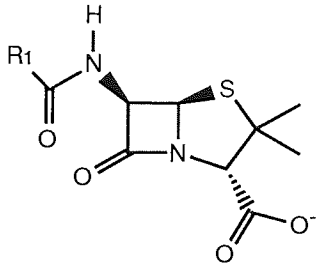
Monolayers were washed (1 x 500 µl x 5 min) with M4. They were incubated with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2 % DMSO at pH 6 in the presence of ACE-NEP inhibitors (for concentration see table). Data are corrected for 15 % non-specific uptake and presented as mean % inhibition ± S.D. for three monolayers and mean IC_{50} ± S.D., with [a], [b] and [c] denoting significant difference at $p < 0.0001$, $p < 0.0005$ and $p < 0.01$, respectively, in % inhibition from the control (uptake in the absence of competitor). y corresponds to a % inhibition value which is too small (% inhibition ≤ 0.00) to be converted into a relevant IC_{50} value.

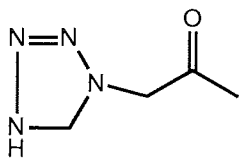
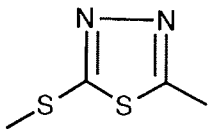
TABLE 6.14 *The effect of a series of cephalosporins on the uptake of Gly-[^{3}H]L-Pro*

Structure A



Structure B



Compound		Conc mM	R_1	R_2	% Inhibition Mean [S.D.]	IC_{50} mM Mean[S.D.]
Cefaclor	A	10	$C_6H_5CH(NH_3^+)$	Cl	68.1 [5.83]^a	4.77[1.33]
Cephradine	A	1	$C_6H_7CH(NH_3^+)$	CH_3	3.72 [5.61]	y
		10			56.5 [2.24]^a	7.72[0.689]
Cephalexin	A	1	$C_6H_5CH(NH_3^+)$	CH_3	0.79 [7.70]	y
		10			51.2 [1.32]^a	9.55[0.511]
Cefazolin	A	10			33.5 [6.90]^b	20.7[5.64]
Ampicillin	B	1	$C_6H_5CH(NH_3^+)$	-	1.73 [4.92]	y
		10			21.6 [6.20]^c	39.0[14.4]
Amoxicillin	B	1	4HO-	-	0.64 [5.67]	y
		10	$C_6H_5CH(NH_3^+)$		19.0 [4.06]^c	44.5[12.9]

Monolayers were washed (1 x 500 µl x 5 min) with M4. They were incubated with M4 containing 50 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro in 2% DMSO at pH 6 or in the presence of 10 mM cephalosporin. Data are corrected for 15% non-specific uptake and presented as mean % inhibition ± S.D. for three monolayers and mean IC_{50} ± S.D. mM, with ^{a}, ^{b} and ^{c} denoting significant inhibition at $p< 0.0001$, $p< 0.01$ and $p< 0.05$, respectively, in % inhibition of the control (uptake in the absence of competitor).

6.4 CONCLUSION

In conclusion, all the ACE-inhibitors tested showed a degree of affinity for the transporter (IC_{50} values of 0.11 ± 0.01 - 32.25 ± 7.17 mM), with the majority of compounds having lower affinities than the natural substrates, the dipeptides (IC_{50} values of 0.10 ± 0.02 - 3.51 ± 0.97 mM). Interestingly, fosinopril, which has a cyclohexyl group at position 4 of the proline ring had a high affinity for the transporter, which is consistent with a previous result shown with the SQ-29852 analogue, SQ-30071 (Chapter 4, Table 4.4). Benazepril, quinapril and SQ-29852, which have all been shown to be substrates for the DTS displayed high affinities for the transporter with IC_{50} values of approximately 1 mM or less [Kim *et al.*, 1994: Hu *et al.*, 1995: Marino *et al.*, 1996: Nicklin *et al.*, 1996]. As expected, the prodrugs benazepril and fosinopril both had higher affinities for the DTS compared to their active moieties. However, this was not the case for enalapril and enalaprilat which showed IC_{50} values which were not quite significantly different. Further investigations exploring the transport mechanisms of the ACE-inhibitors across Caco-2 cell monolayers are required in order to distinguish between a compound being a substrate for the transporter, and thus being translocated into the cell, or simply inhibiting Gly-[^{3}H]L-Pro uptake by blocking the active site on the transporter, as benazeprilat and fosinopril which are reported to be transported v*ia* passive processes, inhibited the probe compound.

The presence of a lipophilic groups substituted onto the seven-membered heterocyclic ring increased affinity in the ACE-NEP inhibitors, indicating that lipophilicity in the region adjacent to the amide nitrogen at the *C*-terminus is important for recognition. Compounds with an increase in the length of the carboxylate chain and atoms substituted in the seven-membered heterocyclic ring still displayed affinity for the DTS. S-S stereochemistry, corresponding to L-L conformation for dipeptides, at the *C* and *N* terminals produced a much greater affinity for the DTS compared to the R-R (D-D) version, as seen with dipeptides. Substitutions which increased affinities for certain compounds, such as the presence of thiol functions, the stereochemistry at the *N*-terminus and a six-membered ring in place of a five-membered ring, were found to have the opposite affect (decrease affinity) for other structures. Thus indicating that the presence of multiple recognition sites on a molecule may override the deleterious effect of any one change. Molecular modelling of selected compounds revealed the possibility of three carbonyl functions oriented in a similar direction that may undergo hydrogen binding with the active site of the transporter.

The β-lactam antibiotics all showed lower affinity for the DTS compared to dipeptides. Comparable results were achieved with cephalexin, cephradine and cefaclor as previously seen in Caco-2 cells. The orally absorbed cephalosporins displayed much higher affinities for the transporter when compared to the parenteral agent cefazolin. The affinities of amoxicillin and ampicillin for the DTS were the same, contradicting a previous study.

CHAPTER 7

INTERACTION OF ODN-CONJUGATES WITH THE DTS IN CACO-2 CELL MONOLAYERS

ABSTRACT

PS ODN conjugated to lipophilic groups either tocopherol (Vitamin E {VitE}) or 2-Di-*O*-hexadecyl-3-glycerol (DHDG), significantly inhibit the functioning of the DTS in cultured Caco-2 intestinal cells. VitE-S-rev inhibited Gly-[^{3}H]L-Pro uptake in a dose-dependent manner, with the maximum inhibition being 36.91 ± 2.13 % at 25 μM, indicating an interaction with the DTS. Greater inhibition by both ODN-conjugates was seen at pH 5, which is consistent with previous data showing increased cell association of ODN at lower pH values [Beck *et al.*, 1996]. Control studies showed no ODN-binding to Gly-[^{3}H]L-Pro and no degradation of ODN during incubation with the cell sheet. Since the DTS mediates the binding and absorption of nutrient peptides and important drugs, such as cephalosporins and penicillin antibiotics, these findings have important implications in relation to the potential toxicity of lipophilic conjugates *in vivo*. It also suggests a potential drug-interaction should modified ODNs be considered for oral delivery.

7.1 BACKGROUND

7.1.1 INTRODUCTION

Antisense ODNs have emerged as novel therapeutic agents for the sequence-specific inhibition of gene expression [Stein & Cheng, 1993] and PS ODNs are currently undergoing clinical evaluation for the treatment of viral infections in AIDS patients and both chronic and acute myelogenous leukaemia's [Agrawal & Akhtar, 1995]. However, the large molecular weight and polar nature of ODNs is a potential limiting factor for therapeutic utilisation. Efficient delivery of ODNs to target tissues remains a problem and a number of delivery strategies, modifications to the ODN structure, and routes of administration [Vlassov *et al.*, 1995] have been examined in order to enhance intracellular bioavailability. The recent demonstration that PO containing segments of 2'-*O*-methyloligoribonucleotide at both 3'- and 5'-ends, are absorbed intact through the GI tract of rats [Agrawal *et al.*, 1995], has highlighted the possibility of oral administration. The aim of this chapter is to explore the interactions of ODNs and ODN conjugated to lipophilic groups, with the GI epithelium, using Caco-2 cells, focusing on possible non-specific interactions with the DTS.

7.1.2 ODN UPTAKE INTO CACO-2 CELLS

Beck *et al.*, have shown that PS and PO both show cellular association with Caco-2 cells, with PS showing greater association than the PO [Beck *et al.*, 1996]. Interactions with the PS were temperature-independent whereas those with the PO were temperature-dependent. Cell-association of ODNs was dependent on length, and at pH 5.5 association resulted in a 2-3 fold increase in binding. PS binding may involve ionic interactions at the cell-surface as NaCl washes removed the ODN in a concentration-dependent manner. Cellular association of both ODNs were susceptible to washing with Pronase (proteolytic enzyme), which accounted for 50 % of interactions. These results suggest that receptor-mediated endocytosis is the major pathway of internalisation for both ODNs in the Caco-2 cells [Akhtar *et al.*, 1996]. This group has also shown that the age of the cells and therefore the extent of differentiation can effect the degree of cellular association, which is probably linked to the levels of binding proteins expressed. A cell-surface binding protein of approximately 46 kDa has been identified as the principle binding site for both ODNs in Caco-2 cells, however binding to a 110 kDa protein was also identified [Akhtar *et al.*, 1996].

7.1.3 ENHANCING INTRACELLULAR BIOAVAILABILITY

A number of approaches have been used in order to enhance intracellular bioavailability. Theses include improving biological stability, improving cellular uptake and, for ODNs taken up by endocytosis, improving efflux from endosomal compartments. Transfer across cell membranes has been improved by conjugating the ODN with lipophilic or hydrophobic groups, such as cholesterol and lipids. Letsinger *et al.* first demonstrated that 5'-cholesteryl-modified PS were more active anti-HIV agents than their unmodified

congeners, but the effect was not sequence-specific [Letsinger *et al.*, 1989]. Increased intracellular accumulation of cholesteryl-conjugates is due to two process; 1) increased absorptive endocytosis by direct insertion of the cholesteryl moiety into the cell membrane [Kreig *et al.*, 1993], and 2) binding to LDL and internalisation *via* the LDL receptor [de Smidt *et al.*, 1991: Kreig *et al.*, 1993]. Although increased cellular uptake has been reported in all incidents with cholesteryl conjugation, the mechanisms of action have not been investigated and a true-antisense effect has not yet been reported [Clarenc *et al.*, 1993].

Lipid conjugation has also demonstrated increased cellular-association. Shea *et al.* have demonstrated greater cell-association (8-10 times) with lipid conjugated ODN in L929 cells compared to the unmodified version [Shea *et al.*, 1990]. Unmodified PO had no activity in a VSV antiviral assay, whereas the lipid-conjugate reduced viral protein synthesis by > 90 %. Antiviral activity was sequence-dependent, however ODNs having little or no base complementarity to the target, were still effective [Shea *et al.*, 1990]. *N*-[1(-2, 3-dioleyloxy)propyl]-n$_1$n$_1$n-trimethylammonium chloride (Lipofectin), is a synthetic cationic lipid, which has been shown not only to increase cellular uptake, but also accumulation of the ODN in the nucleus [Bennett *et al.*, 1993]. Other examples of conjugation include biotinylation ODNs complexed to avidin (a cationic protein) which enhances cellular uptake by increasing binding to the negatively charged surface of the cell [Pardridge & Boado, 1991], and conjugation with neoglycoproteins, which have been shown to increase uptake into macrophages [Clarenc *et al.*, 1993]. Attaching a hydrophobic undecyl residue at the 5'-end of D-oligo (PO) targeted against the influenza virus has produced sequence-specific inhibition of viral replication, indicating enhanced bioavailability [Kabanov *et al.*, 1990].

Poly-L-lysine modifications are thought to increase cell uptake by masking the negative charge of the ODN and also allow the ODN to escape from the endosomal compartments by destabilising the membranes in question [Kregenow *et al.*, 1995]. Poly-L-lysine conjugated ODNs have also been shown to produced sequence-specific effects. However, poly-L-lysine is toxic to cells, thus complexing of the ODN conjugate with heparin, to reduce cellular toxicity, has been investigated in order to reduce cellular toxicity [Degols *et al.*, 1991]. Conjugation to antibodies raised against receptors present on the apical membrane is another approach being used to increase cellular association. The cellular delivery of transferrin receptor antibody-ODN conjugate has been investigated in the human glioblastoma cell line, U87-MG and the human endothelial cell line, ECV304, where cellular association was found to be 3-fold higher than with free ODN [Walker *et al.*, 1995]. Conjugation of ODN to a EGF-poly-L-lysine complex has also demonstrated increased uptake *via* EGF-receptor mediated endocytosis [Deshpande *et al.*, 1996].

The oral route of delivery for ODNs is somewhat limited by the fact that enzymatic hydrolysis may occur along the GI tract, thus limiting delivery to the target site. Incorporation of the ODN into liposomes is a possible delivery strategy. Liposomes offer a biocompatible and biodegradable system and ensure cellular uptake *via* fusion with cell membranes. They also protect the ODN from enzymatic degradation and offer the

possibility of a controlled release system, as efflux appears to be slow and sustainable over several days [Akhtar *et al.*, 1991a]. Therefore, the sustained release makes them suitable for genetic targets with slow turnover. Targeting liposomes to cell surfaces using antibodies has also been suggested [Leonetti *et al.*, 1990]. Liposomes are already undergoing clinical trials for treatment of diseases such as infections by intracellular parasites and have been approved for a trial as gene transporters into tumour cells [Clarenc *et al.*, 1993].

7.2 MATERIALS AND METHODS

7.2.1 MATERIALS

Details of suppliers for the cell culture materials used throughout this chapter have been previously described (section 2.1-2.1.3). The ABI 392 automated DNA synthesiser was purchased from Applied BioSystems (Warrington, UK) and the NENSORB 20 column was from Dupont NEN Research Products (Hertfordshire, UK). Materials used for gel electrophoresis were purchased from Sigma Chemical Co. (Poole, Dorset, UK).

7.2.2 METHODS

7.2.2.1 MEDIA

Details of cell growth media, M1 and M4 (pH 6, unless otherwise stated) used throughout this chapter have been previously described (section 2.2.1.1).

7.2.2.2 CELL CULTURE

Cell line-1 (section 2.1.1)was used between passage number 25-38. The cells were seeded onto 24-well plates (2 cm^2) at a density of 1.6 x 10^5 cells $well^{-1}$ and grown in an atmosphere of 5 % CO_2 (95 % air) and 90 % relative humidity, at 37°C, as previously described (section 2.2.1.3). M1 (2 ml) was renewed every 48 h and the monolayers were used after 7 d growth.

7.2.2.3 ODN SYNTHESIS

The unconjugated ODN were synthesised by Dr. D. Dunnion, Aston University, UK. The 28-mer PS ODN (S-rev; 5' TCG TCG CTG TCT CCG CTT CTT CCT GCC A 3') complementary to the *rev* gene in HIV-1 was synthesised on an ABI 392 automated DNA synthesiser using standard phosphoramidite chemistry, with tetraethyl thiuram disulphide as the sulphurising reagent. 10 pmols S-rev was 5'-end labelled with [γ^{32}P]-dATP using T4 polynucleotide kinase, as previously described [Akhtar *et al.*, 1991a: Akhtar *et al.*, 1996: Beck *et al.*, 1996]. The unincorporated label was removed on a NENSORB 20 column according to the manufacturer's protocol. Labelled ODNs were made up to 10 μM with the corresponding unlabelled ODN before use. The ODN-conjugates, Vitamin E-S-rev (VitE-S-

rev) and 2-Di-*O*-hexadecyl-3-glycerol-S-rev (DHDG-S-rev) conjugates (Figure 8.1) were synthesised as previously described by Professor T. Brown [Will & Brown, 1992: Mackellar *et al.*, 1992]. The ODN-conjugates were purified by HPLC on a Brownlee Aquapore small rp 300 C8 (octyl) reversed-phase column using a gradient of 0-60 % (v/v) acetonitrile in a buffer of 0.1 *M* ammonium acetate. After HPLC, solvent was removed on a rotary evaporator and the ODN-conjugate was dissolved in 1 ml of double distilled water and freeze-dried. It was then desalted by Sephadex G25 gel-filtration.

7.2.2.4 EXPERIMENTAL CONDITIONS

7.2.2.4.1 Gly-[^{3}H]L-Pro Studies

The following general experimental procedure was used throughout this chapter, unless otherwise stated. All experimental solutions were preincubated at 37°C prior to experimentation, with the exception of PBS-azide which was kept at 4°C. The maintenance media were aspirated and the monolayers washed with M4 at pH 6 (1 x 500 µl x 10 min) at 37°C, unless otherwise stated. The incubation solution (M4 at pH 6) contained 62.5 nM of Gly-[^{3}H]L-Pro, 10 mM L-Pro and the required amount of ODN/ODN-conjugate (for details see individual experiments). Two different incubation procedures were used: 1) (1 x 200 µl x 3 min) at 37°C and 2) pre-incubation (1 x 200 µl x 15 min) with incubation solution in the absence of the probe at 37°C, and then addition of 62.5 nM Gly-[^{3}H]L-Pro to each well and further incubation for 3 min, at 37°C. Apical solutions were collected, and plates transferred to the cold-table (4°C) and washed with ice-cold PBS-azide solution (2 x 500 µl x 5 min). The monolayers were harvested by solubilising with 1 ml of 1 % v/v TX-100 in double distilled water. Apical solutions, washings and solubilised cells were collected made up to 1 ml with double distilled water, added to 10 ml of HiSafe 3 and counted for ^{3}H-content by LSC.

7.2.2.4.2 [^{32}P]-S-rev Studies

The experimental conditions used were the same as described in section 7.2.2.4.1 with the following exceptions; 1) The incubation solution contained trace elements of [^{32}P]-S-rev which was mixed with 10 µM unlabelled S-rev, in M4 at pH 6, and 2) the incubation procedure was (1 x 200 µl x 18 min) at 37°C. Solutions were counted for ^{32}P-content by LSC. [^{32}P]-S-rev studies were carried out by Dr. D. Dunnion, Aston University, UK.

7.2.2.5 STABILITY OF ODN

The gel electrophoresis were carried out by Dr. D. Dunnion, Aston University, UK. An electrophoresis gel was run to assess degradation of the labelled ODN (presence of free label, [γ^{32}P]-dATP). Samples were mixed with formamide loading buffer (80 % formamide, 10 mM EDTA at pH 8.0, 1 mg m^{-1} xylene cyanol FF and 1 mg ml^{-1} bromophenol blue) and

stored at -20 °C. These were heated to 100 °C for 5 min and separated on 7 *M* urea / 20 % acrylamide gels: bands were then detected by autoradiography of wet gels.

7.2.3 THE EFFECT OF ODN AND ODN-CONJUGATES ON THE UPTAKE OF GLY-[^{3}H]L-PRO INTO CACO-2 CELL MONOLAYERS

7.2.3.1 THE EFFECT OF ATP AND SQ-29852 ON THE UPTAKE OF GLY-[^{3}H]L-PRO INTO CACO-2 CELL MONOLAYERS

To assess whether mononucleosides interact with the DTS, the effect of ATP on the uptake of Gly-[^{3}H]L-Pro was investigated. SQ-29852 was included as a positive control to check the reproducibility of the system. The cells were seeded onto 24-well plates as previously described (section 2.2.1.3), and used 7 d post-seeding. The incubation solution contained 62.5 nM Gly-[^{3}H]L-Pro with 10 mM L-Pro, in M4 at pH 6 (control), and in the presence of 1 mM ATP and 1 mM SQ-29852. The incubation period was 3 min.

7.2.3.2 THE EFFECT OF ODN AND ODN-CONJUGATES ON THE UPTAKE OF GLY-[^{3}H]L-PRO INTO CACO-2 CELL MONOLAYERS

The cells were seeded onto 24 well-plates as previously described (section 2.2.1.3), and used 7 d post-seeding. The incubation solution contained 62.5 nM Gly-[^{3}H]L-Pro with 10 mM L-Pro, in M4 at pH 6 (control). The following ODN and ODN-conjugates were co-administered with the control solution at the given concentration; S-rev at 25 μM, Vitamin E (VitE) at 25 μM, DHDG-S-rev at 25 μM, VitE-S-rev at 1, 10 and 25 μM. Uptake was investigated for both incubation procedures, (1 x 200 μl x 3 min) and pre-incubation (1 x 200 μl x 15 min) in the absence of the probe, than addition of the Gly-[^{3}H]L-Pro to the well, followed by a further 3 min incubation, at 37°C.

7.2.3.3 THE EFFECT OF ODN AND ODN-CONJUGATES ON THE UPTAKE OF GLY-[^{3}H]L-PRO INTO CACO-2 CELL MONOLAYERS AT pH 5

The effect of pH on the interaction of the ODN and ODN-conjugates with the DTS was investigated. The cells were seeded onto 24-well plates as previously described (section 2.2.1.3), and used 7 d post-seeding. The monolayers were washed (1 x 500 μl x 30 min) at 37°C, with M4 at pH 5 (M4 at pH 6 was used for control-2). The incubation solution contained 62.5 nM Gly-[^{3}H]L-Pro with 10 mM L-Pro, in M4 at pH 5 (control-1). The following ODN and ODN-conjugates were co-administered with the control solution at a concentration of 25 μM; S-rev, VitE, DHDG-S-rev and VitE-S-rev. Uptake of the control was also studied at pH 6 (control-2). The incubation procedure was as follows; pre-incubation (1 x 200 μl x 15 min) in the absence of the probe, than addition of the Gly-[^{3}H]L-Pro to the well and incubated for a further 3 min, at 37°C.

7.2.3.4 THE EFFECT OF SQ-29852 AND VITE-S-REV ON THE UPTAKE OF GLY-[^{3}H]L-PRO INTO CACO-2 CELL MONOLAYERS, WHEN CO-ADMINISTERED TOGETHER

The cells were seeded onto 24-well plates as previously described (section 2.2.1.3), and used 7 d post-seeding. The incubation solution contained 62.5 nM Gly-[^{3}H]L-Pro with 10 mM L-Pro, in M4 at pH 6 (control). Uptake was investigated in the presence of 1 mM SQ-29852, and 1 mM SQ-29852 with 25 μM VitE-S-rev. Monolayers were pre-incubated (1 x 200 μl x 15 min) at 37°C, with the incubation solution in the absence of the probe. The probe was then added to each well and monolayers were incubated for 3 min, at 37 °C.

7.2.3.5 THE EFFECT OF ODN AND ODN-CONJUGATES ON THE BINDING OF [^{32}P]-S-REV TO CACO-2 CELL MONOLAYERS AT pH 5 OR pH 6

The cells were seeded onto 24-well plates as previously described (section 2.2.1.3), and used 7 d post-seeding. The monolayers were washed (1 x 500 μl x 30 min) at 37°C, with M4 at the appropriate pH (5 or 6). Monolayers were then incubated with M4 containing [^{32}P]-S-rev alone, or in the presence of one of the following compounds at 25 μM; S-rev, Gly-L-Pro, VitE, DHDG-S-rev and VitE-S-rev all at pH 6, and [^{32}P]-S-rev alone and in the presence of 25 μM DHDG-S-rev at pH 5. The incubation period was (1 x 200 μl x 18 min) at 37°C. Results are expressed as % inhibition for the appropriate control.

7.2.3.6 ODN STABILITY

7.2.3.6.1 Is the ODN binding to the probe (Gly-[^{3}H]L-Pro)

Due to the small quantity of S-rev available, the preliminary stability and binding studies were carried out using [^{32}P]-S-tat. [^{32}P]-S-tat was incubated with 62 nM Gly-[^{3}H]L-Pro in M4 at pH 6 containing 10 mM L-Pro. Samples were taken after 3, 15 and 18 min incubation at 37°C. The control consisted of [^{32}P]-S-tat in M4 at pH 6 containing 10 mM L-Pro at the start of the experiment (0 min). Samples were assessed for [^{32}P]-S-tat binding to the probe, by gel electrophoresis, as previously described (section 7.2.2.5). A visible shift in the control band would correspond to binding to the probe or components of the incubation media. Free [γ^{32}P]-dATP was also included to monitor any degradation of the [^{32}P]-S-tat during the experiment.

7.2.3.6.2 S-tat stability when incubated with Caco-2 cell monolayers

[^{32}P]-S-tat, in M4 at pH 6 containing 10 mM L-Pro was incubated (1 x 250 μl) with a cell sheet (24-well plate) at 37°C for 3, 15 and 18 min. The controls consisted of [^{32}P]-S-tat, in M4 at pH 6 containing 10 mM L-Pro, and [γ^{32}P] alone. Samples were assessed for [^{32}P]-S-tat degradation, by gel electrophoresis, as previously described (section 7.2.2.5).

7.2.3.6.3 S-rev stability when incubated with Caco-2 cell monolayers

The above experiment (8.2.3.6.2) was repeated as described with S-rev replacing the S-tat.

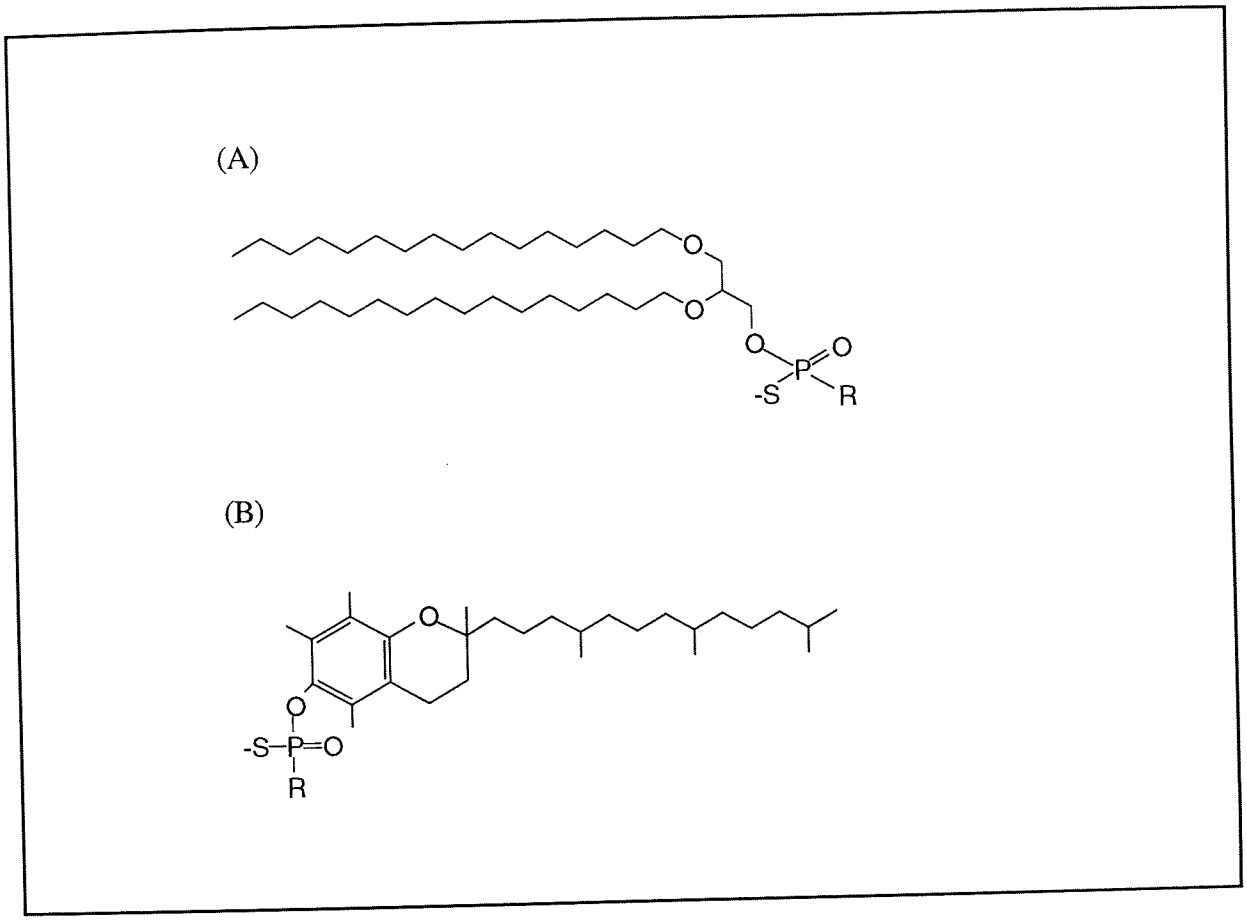
7.3 RESULTS AND DISCUSSION

The generally inefficient uptake of unconjugated ODN by endocytosis has led to the investigation of alternative delivery strategies for ODN, such as the use of lipophilic conjugates [Vlassov *et al.*, 1995]. The aim of this study was to investigate the likely non-specific interactions of lipophilic VitE-S-rev and DHDG-S-rev conjugates (Figure 7.1), which may influence the efficiency of uptake mechanisms of nutrient dipeptides or drugs *via* the DTS in Caco-2 cells.

A 10 min pre-washing period was selected for all experiments. The maintenance medium M1 contains 10 % FBS, which may degrade the ODN [Akhtar *et al.*, 1991b] therefore, to ensure that all FCS is washed from the cell surface, the washing protocol was increased from 5 min (previously used) to 10 min. For experimental studies where there is a change in pH, the pre-washing period is 30 min, to allow for cells to equilibrate to the change in environment.

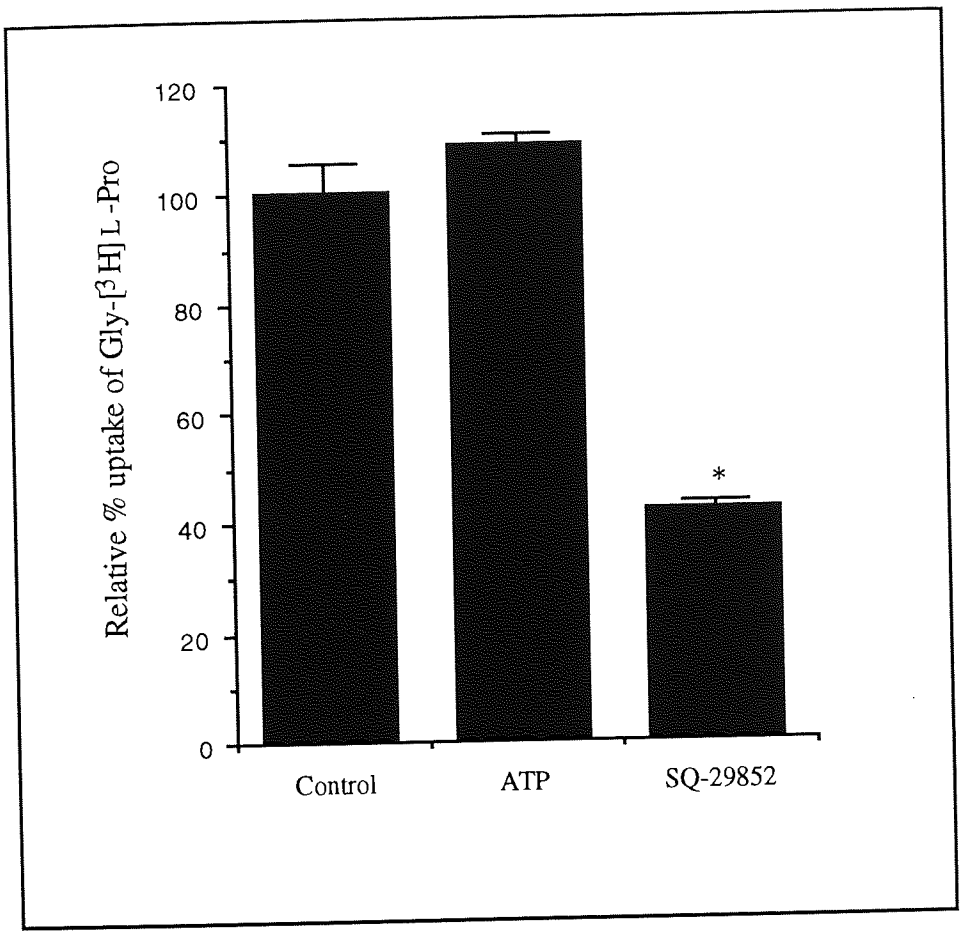
The uptake of Gly-[^{3}H]L-Pro into Caco-2 cells monolayers was unaffected by the presence of 1 mM ATP (Figure 7.2), confirming that mononucleotides are not substrates for the DTS, nor exert non-specific interactions with the transporter. SQ-29852 produced a significant reduction in uptake of the probe, and at a concentration of 1 mM produced 59.98 ± 1.24 % inhibition, which agrees with the previous results of 60.51 ± 6.73 % (section 3.2.2), indicating that the system is reproducible between studies.

FIGURE 7.1 *Structures of lipophilic-ODN conjugates*



The lipophilic moieties (A) = 2-Di-*O*-hexadecyl-3-glycerol or (B) = Vitamin E (tocopherol) were conjugated to a 28-mer-phosphorothioate ODN (S-rev) *via* its 5'-terminal thiophosphate where R is the ODN, 5' TCG TCG CTG TCT CCG CTT CTT CCT GCC A 3'.

FIGURE 7.2 *The effect of ATP and SQ-29852 on the uptake of Gly-[^{3}H]L-Pro into Caco-2 cell monolayers*



Monolayers were washed (1 x 500 µl x 10 min) at 37°C, with M4 at pH 6, then incubated (1 x 200 µl x 3 min) at 37°C with M4 containing 62.5 nM Gly-[^{3}H]L-Pro and 10 mM L-Pro (Control), 1 mM ATP and 1 mM SQ-29852. Data are expressed as mean (of at least three monolayers) ± S.D. of % inhibition from the control value. * Denotes a significant ($p < 0.05$) reduction from the control .

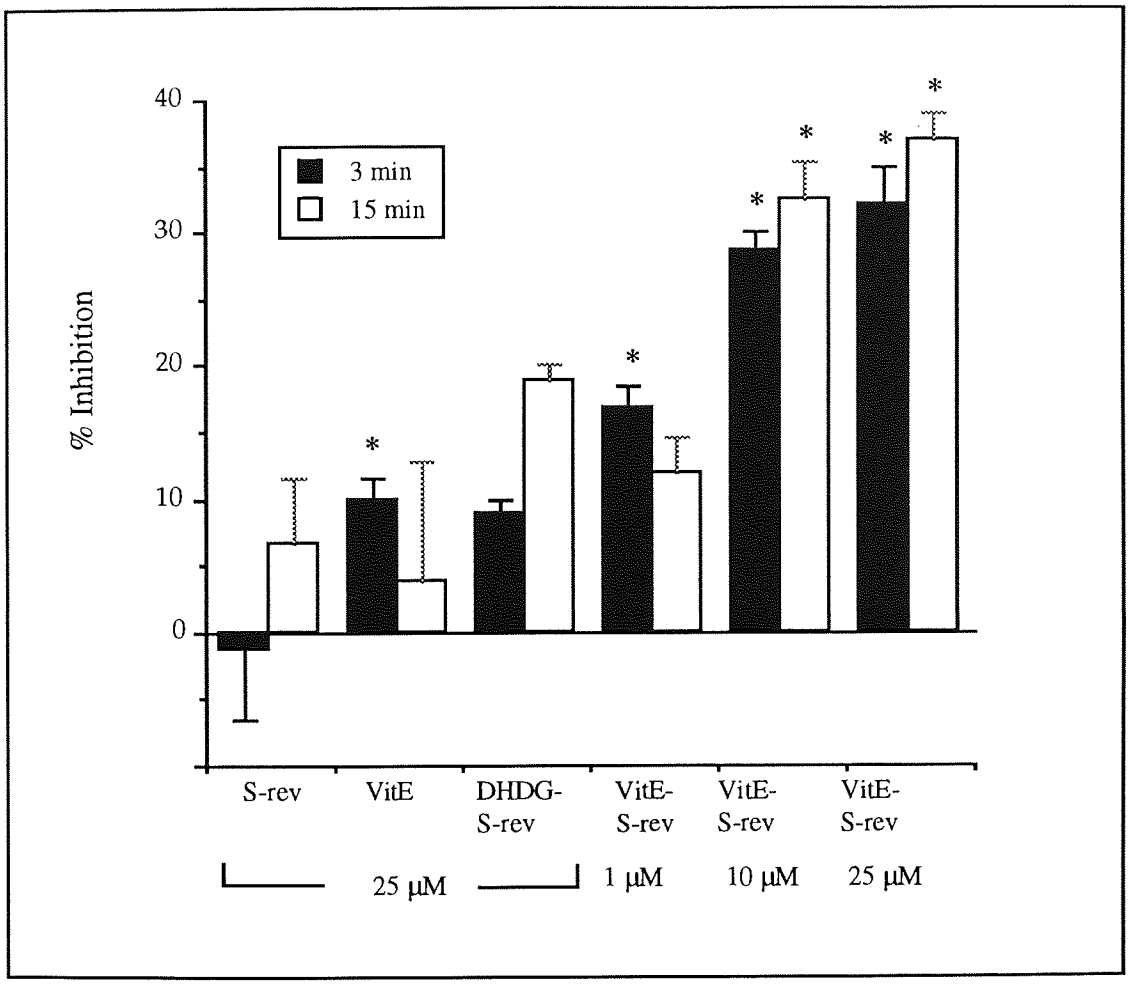
Cellular association of PS ODN with Caco-2 cells has previously been shown to be biphasic, with a rapid initial phase being complete within 15 min [Beck *et al.*, 1996]. Therefore, uptake was investigated after 3 min and after a 15 min pre-incubation period with the ODN, to allow for binding. Unconjugated S-rev had no significant effect on uptake of the probe at 25 μM (Figure 7.3), illustrating that the ODN has no interaction with the DTS at 25 μM. DHDG-S-rev produced 18.99 ± 1.08 % inhibition of Gly-[^{3}H]L-Pro uptake. However, this was not quite significantly different from the control value at the confidence limits tested (95 %), suggesting that DHDG-S-rev has no interaction with the DTS. VitE-S-rev produced a dose-dependent inhibition (up to 36.91 ± 2.13 % at 25 μM) at pH 6. Results for the two incubation procedures for VitE-S-rev were not significantly different, indicating that interaction with the DTS is occurring within the 3 min period. Little or no significant inhibition was observed with unconjugated S-rev ODN or VitE alone, suggesting that the inhibitory effect required the ODN-VitE lipophilic conjugate.

Inhibition of Gly-[^{3}H]L-Pro uptake by the ODN-conjugates was increased dramatically at pH 5 (Figure 7.4), however, the unconjugated ODN, S-rev, and the parent lipophilic moiety, VitE, still had no effect on uptake at pH 5. VitE-S-rev and DHDG-S-rev produced 77.33 ± 5.10 and 72.70 ± 9.06 % inhibition respectively. Note that the increase in inhibition is much greater for the DHDG-S-rev (18.99 ± 1.08 % at pH 6 to 72.70 ± 9.06 % inhibition at pH 5) than that seen for the VitE-S-rev. However, the inhibition seen with the ODN-conjugates were not significantly different from one another. PS ODN bind more avidly to Caco-2 cells with decreasing pH , and the optimal pH for ODN binding has been shown to be pH 5 [Beck *et al.*, 1996]. Therefore, increased inhibition of the probe, may be explained by enhanced cell surface binding of the ODN-conjugates at pH 5. The predicted Hansch Log P values for VitE and DHDG moieties are 9.6 and 11.1 respectively [Hansch & Leo, 1979]. Although these molecules are highly lipophilic, their ionic character is unlikely to change over the pH range studied. The decrease in pH will not affect the ODN or its conjugate, but it may possibly protonate the cell surface proteins, making them more attractive to the negatively charged ODN [Beck *et al.*, 1996]. The α-amino group of lysine, the guanidium group of arginine and the protonated imidazole of histidine are protein sites that have been suggested in ODN binding [Blackburn & Gait, 1990]. Histidine, having a pKa of 6.5 is susceptible to protonation over a pH range of 7.2 to 5.0. Histidyl residues have been reported to be associated with the DTS in [Kato *et al.*, 1989: Kramer *et al.*, 1993]. Therefore, the enhanced affinity of the ODN-conjugates at pH 5 may possibly be due to protonation of histidine residues present at the DTS binding site.

Uptake of Gly-[^{3}H]L-Pro was reduced by 29.11 ± 3.26 % at pH 5 compared to that at pH 6 (data not shown), which is in agreement with previous results (section 3.3.1). The optimum pH for the DTS is pH 6, providing an inward proton gradient to fuel the H^+ cotransport system [Thwaites *et al.*, 1993a, b, c, g, 1994a]. Previous data indicated a 42.95 ± 8.35 % reduction in total uptake when the pH is changed from 6 to 5. However, the discrepancy between results may be explained by the fact that the previous study (section

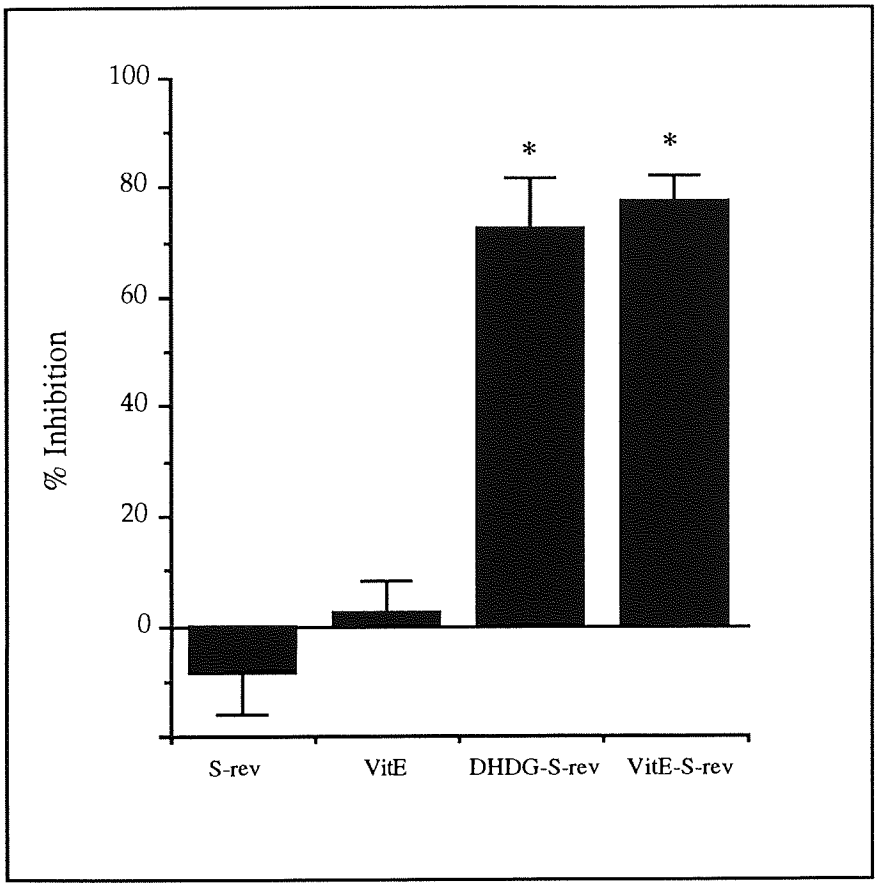
3.3.1) was carried out over a 3 min incubation period, whereas the present study is over an 18 min period, with a 15 min pre-incubation step in absence of the probe. Therefore, as experimental conditions are not constant, direct comparisons can not be made.

FIGURE 7.3 *The effect of ODN and ODN-conjugates on the uptake of Gly-[^{3}H]L-Pro into Caco-2 cell monolayers*



The monolayers were washed (1 x 500 µl x 10 min) with M4 at pH 6, and then incubated (1 x 200 µl x 3 min, solid bar) at 37°C with M4 containing 62.5 nM of Gly-[^{3}H]L-Pro and 10 mM L-Pro, in the presence of one of the following compounds; 25 µM S-rev, 25 µM VitE, 25 µM DHDG-S-rev, and 1, 10, and 25 µM VitE-S-rev, all at pH 6. The experiment was repeated using a pre-incubation step (white bar) where the monolayers were incubated (1 x 200 µl x 15 min) at 37°C with M4 containing 10 mM L-Pro and the appropriate compounds, followed by addition of 62.5 nM of Gly-[^{3}H]L-Pro to each well and then incubated for a further 3 min period at 37°C. Data are expressed as mean (of at least three monolayers) ± S.D. * Denotes a significant reduction ($p < 0.05$) from the appropriate control values (Gly-[^{3}H]L-Pro uptake after 3 min and after 15 min pre-incubation and then 3 min with the probe).

FIGURE 7.4 *The effect of ODN and ODN-conjugates on the uptake of Gly-[^{3}H]L-Pro into Caco-2 cell monolayers at pH 5*



The monolayers were washed (1 x 500 µl x 30 min) with M4 at pH 5 at 37°C. Monolayers were then pre-incubated (1 x 200 µl x 15 min) with M4 at pH 5 containing 10 mM L-Pro, and 25 µM of one of the following compounds; S-rev, VitE, DHDG-S-rev and VitE-S-rev. The probe (62.5 nM Gly-[^{3}H]L-Pro) was then added to each well and the monolayers incubated for a further 3 min at 37°C. The data are expressed as mean (of at least three monolayers) ± S.D. * Denotes a significant reduction ($p < 0.05$) from the control value (uptake of Gly-[^{3}H]L-Pro at pH 5).

Co-administration of 1 mM SQ-29852 and 25 μM VitE-S-rev produced 49.54 ± 6.36 % inhibition of Gly-[^{3}H]L-Pro uptake (Figure 7.5). The inhibition observed was not significantly different from that seen when administering SQ-29852 alone (57.13 ± 3.09 %). Therefore, we are not seeing an additive effect and results suggests that VitE-S-rev inhibits DTS function by binding to the same sites (on the DTS) as SQ-29852, or that SQ-29852 has a greater affinity for these sites than the ODN-conjugate. Pre-incubation for 15 min with SQ-29852 had no significant effect on the % inhibition observed previously with the 3 min incubation period (57.98 ± 1.24 %).

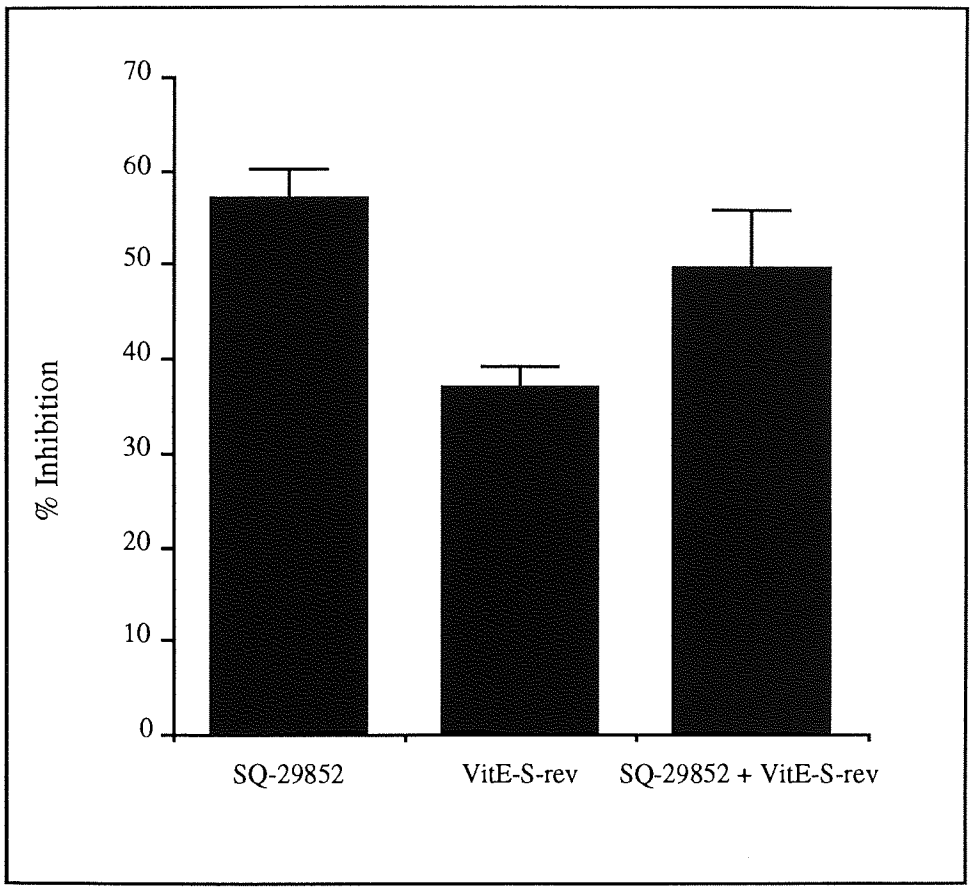
Radiolabelled conjugates were unavailable for direct cell-binding studies. The lipophilic moieties occupied the 5'-end and therefore could not be labelled at this position. Labelling at the 3'-end was not practical as previous results had shown that they are not good substrates for terminal transferase (oral communication, Akhtar 1996). The relative binding affinities of the ODN-conjugates were investigated by their ability to compete for binding sites occupied by radiolabelled unconjugated S-rev.

All compounds tested produced significant inhibition of [^{32}P]-S-rev association with the cell monolayers, with the exception of VitE, which correlates well with the lack of inhibition seen with Gly-[^{3}H]L-Pro uptake by the lipophilic parent molecule (Figure 7.6). 25 μM of unlabelled S-rev produced 75.30 ± 0.34 % inhibition. However, VitE-S-rev had a much greater effect producing 94.33 ± 0.11 % inhibition, which is indicative of improved cell-surface binding of the ODN-conjugate. The greater affinity of VitE-S-rev for cell-surface binding ODN binding sites in Caco-2 cells is consistent with the greater inhibition of the DTS by this conjugate. This is further supported by the fact that the DHDG-S-rev conjugate produced a lower inhibition (38.36 ± 3.31 %) than the VitE-S-rev. However, at pH 5, DHDG-S-rev showed an improved affinity for S-rev ODN binding-sites (58.36 ± 6.47 %) which is consistent with increased binding at lower pH [Beck *et al.*, 1996]. Gly-L-Pro produced a slight inhibition of 27.11 ± 2.49 % . This was an unexpected result as S-rev did not effect uptake of Gly-[^{3}H]L-Pro.

The results suggest that unconjugated S-rev has no interaction with the DTS in Caco-2 cells, whereas ODN conjugated to lipophilic moieties, such as VitE, are capable of interaction with the DTS. Akhtar *et al.*, have reported that PS ODN bind to proteins of 30, 34, 46 and 110 kDa, with predominant binding being seen at the 46 kDa cell-surface protein [Akhtar *et al.*, 1996]. The identity of this latter protein is not known, but a protein of 120 ± 10 kDa, which has been shown to be directly involved in the uptake of small peptides, has been isolated from the membranes of Caco-2 cells [Dantzig *et al.*, 1994b]. The improved binding of the VitE-S-rev for S-rev binding-sites may represent an enhanced affinity of the conjugate for this protein. The DHDG-S-rev conjugate displayed much lower competition for the free ODN (S-rev), suggesting that it is binding to different sites on the cell surface. These may involve both proteins [Akhtar *et al.*, 1996] and lipid binding sites, as some ODN derivatives interact with lipid vesicles [Akhtar *et al.*, 1991a]. VitE-S-rev appears to have a greater affinity for interaction with the DTS, or possibly to surface proteins and/or lipids

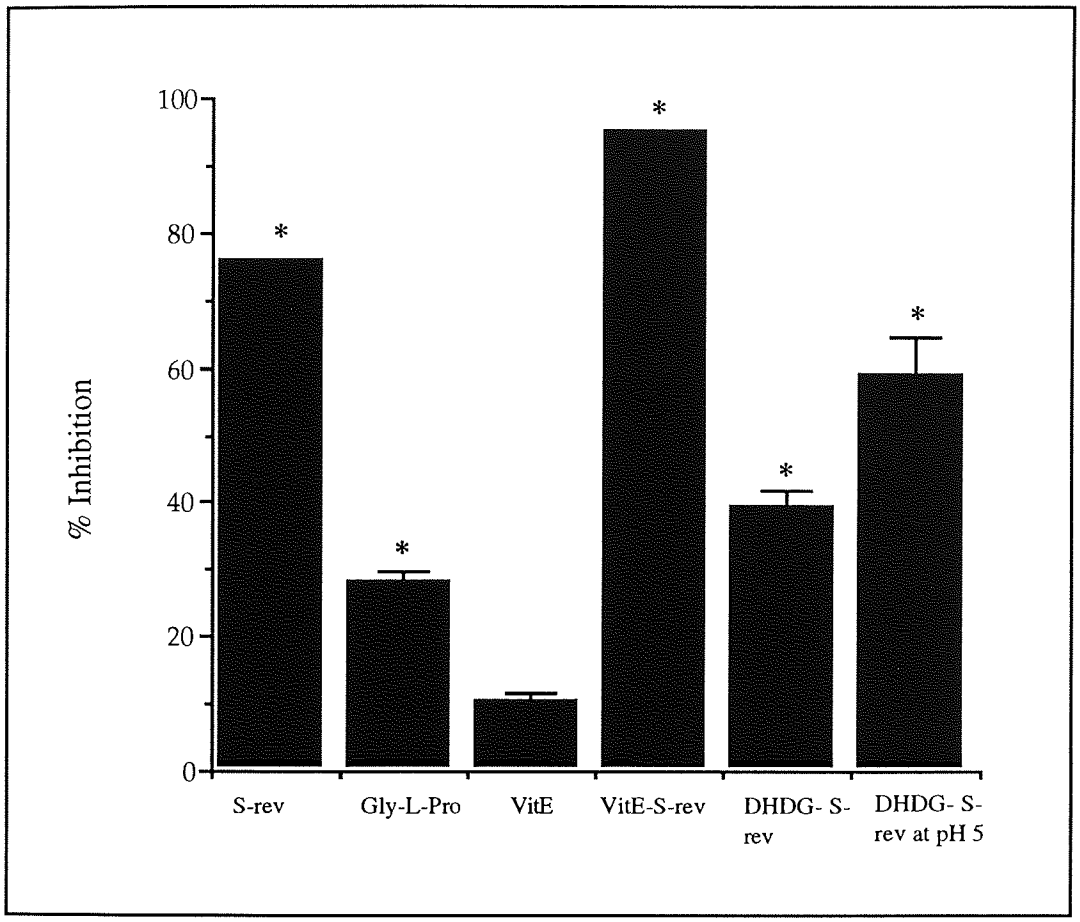
regulating the functioning of the DTS (allosteric inhibition) than DHDG-S-rev, which showed no significant effect at pH 6. At pH 5, equal capacity for interaction with the DTS by the ODN conjugates was exhibited, indicating an enhanced affinity for cell surface sites regulating the DTS.

FIGURE 7.5 *The effect of SQ-29852 and VitE-S-rev on the uptake of Gly-[^{3}H]L-Pro into Caco-2 cell monolayers, when co-administered together*



The monolayers were washed (1 x 500 µl x 10 min) with M4 at pH 6 at 37°C. Monolayers were then incubated (1 x 200 µl x 15 min) at 37°C, with M4 containing 10 mM L-Pro at pH 6, in the presence of 1 mM SQ-29852, 25 µM VitE-S-rev and 1 mM SQ-29852 together with 25 µM VitE-S-rev. 62.5 nM of Gly-[^{3}H]L-Pro was then added to each well, and the monolayers were incubated for a further 3 min at 37°C. Data are expressed as mean (of at least three monolayers) ± S.D.

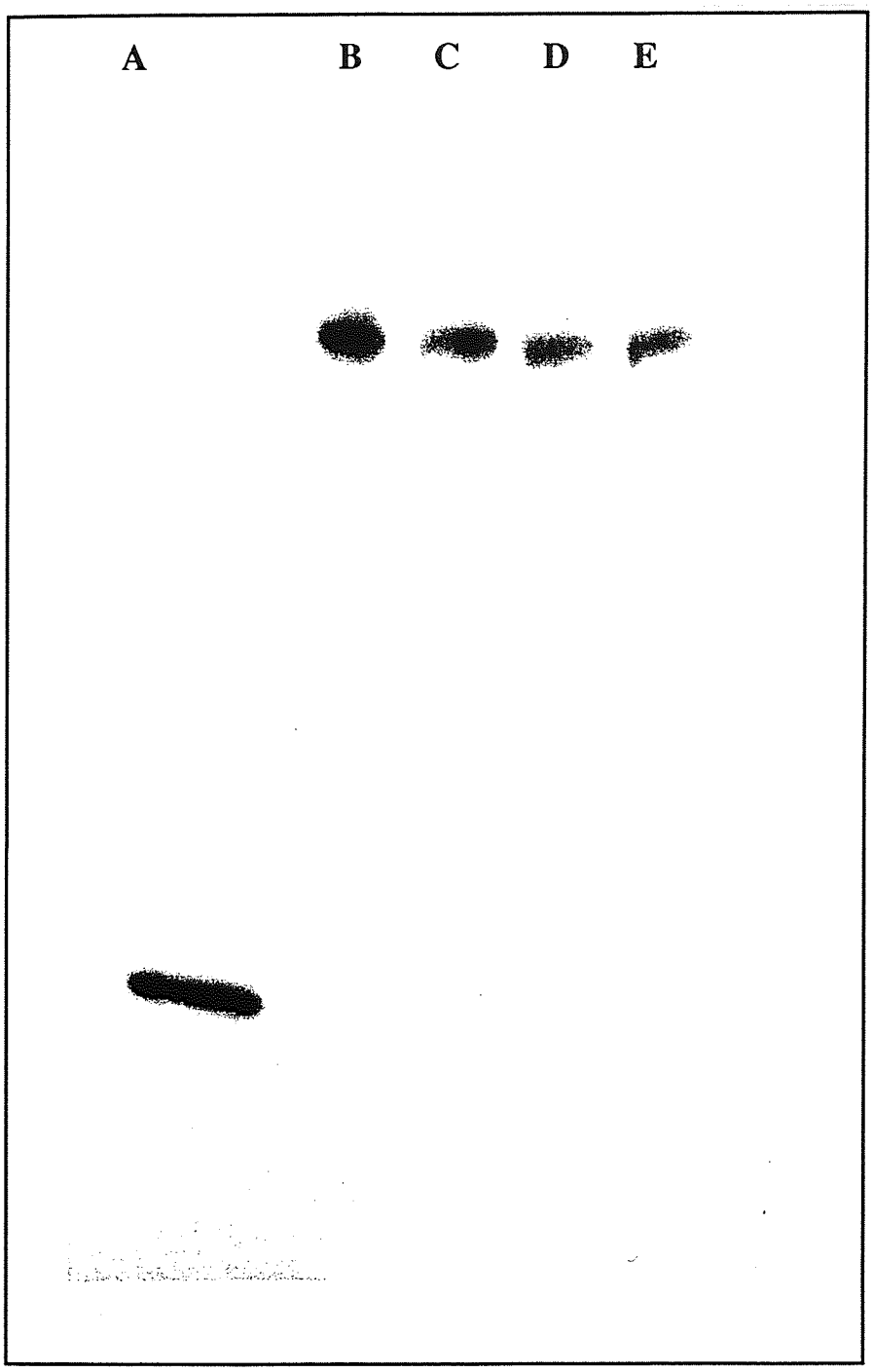
FIGURE 7.6 *The effect of ODN and ODN-conjugates on the binding of [^{32}P]-S-rev to Caco-2 cell monolayers at pH 5 or pH 6*



The monolayers were washed (1 x 500 µl x 30 min) with M4 at the appropriate pH, at 37°C. The monolayers were then incubated (1 x 200 µl x 18 min) at 37°C with M4 containing 5' end radiolabelled [^{32}P]-S-rev in the presence of 25 µM of the following compounds; S-rev, Gly-L-Pro, VitE, VitE-S-rev and DHDG-S-rev, all at pH 6. The control ([^{32}P]-S-rev alone) and 25 µM DHDG-S-rev were also studied at pH 5. Data are expressed as mean (of at least three monolayers) ± S.D. * Denotes a significant reduction from the appropriate control value (pH 5 or pH 6).

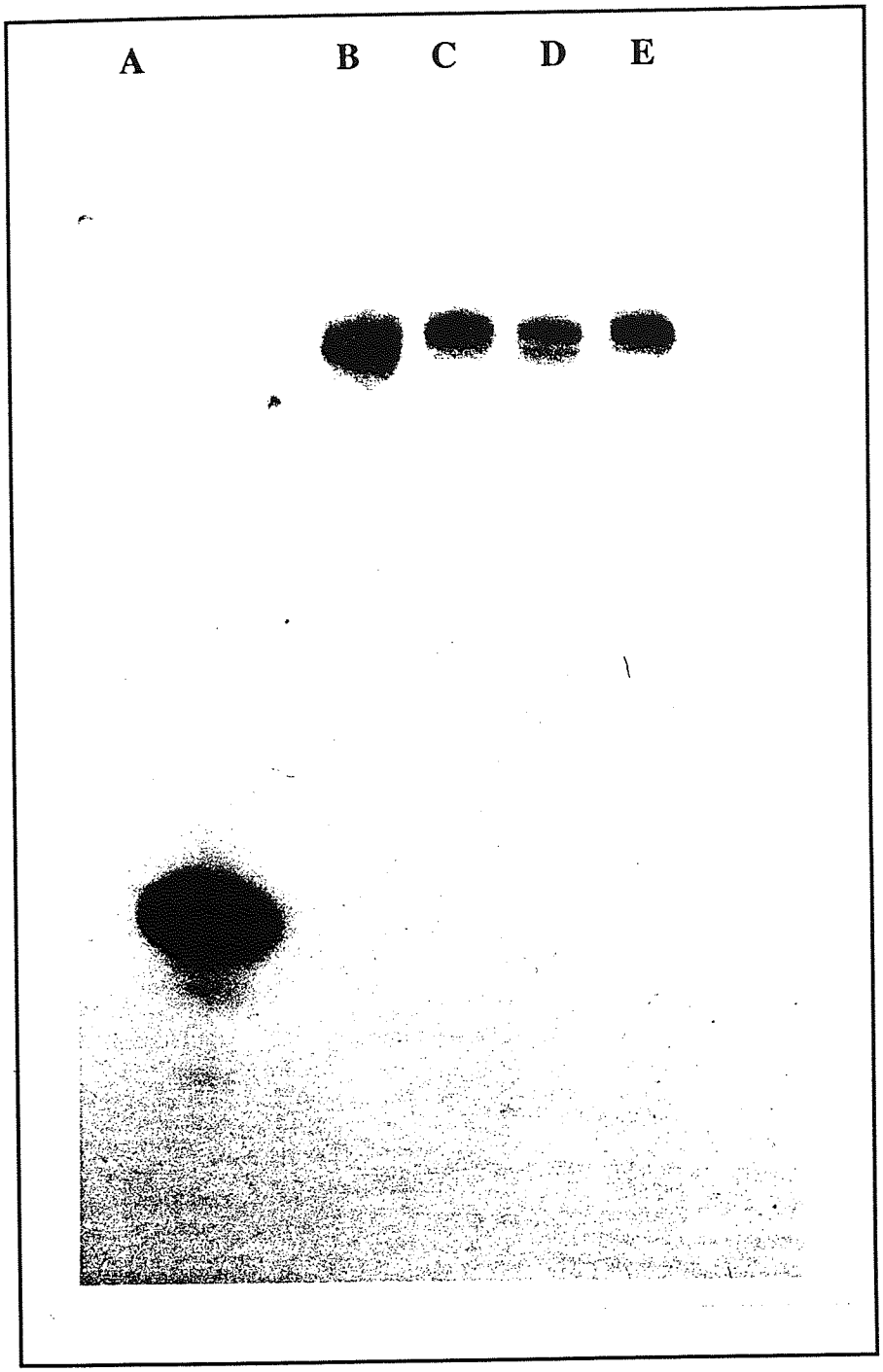
The binding study for [^{32}P]-S-tat revealed that the ODN was not binding to the probe as no band shift was observed (Figure 7.7). Both [^{32}P]-S-tat and [^{32}P]-S-rev were stable when incubated with the cell monolayers. No degradation was occurring as free label, [γ^{32}P]-dATP, was not visible with the [^{32}P]-S-tat and [^{32}P]-S-rev samples (Figure 7.8, Figure 7.9), after the required time periods. B-E points on Figure 7.9 appear to be moving slightly downwards, however this is due to the angle at which the gel was run at.

FIGURE 7.7 *[^{32}P]-S-tat stability in incubation media with the probe, Gly-[^{3}H]L-Pro*



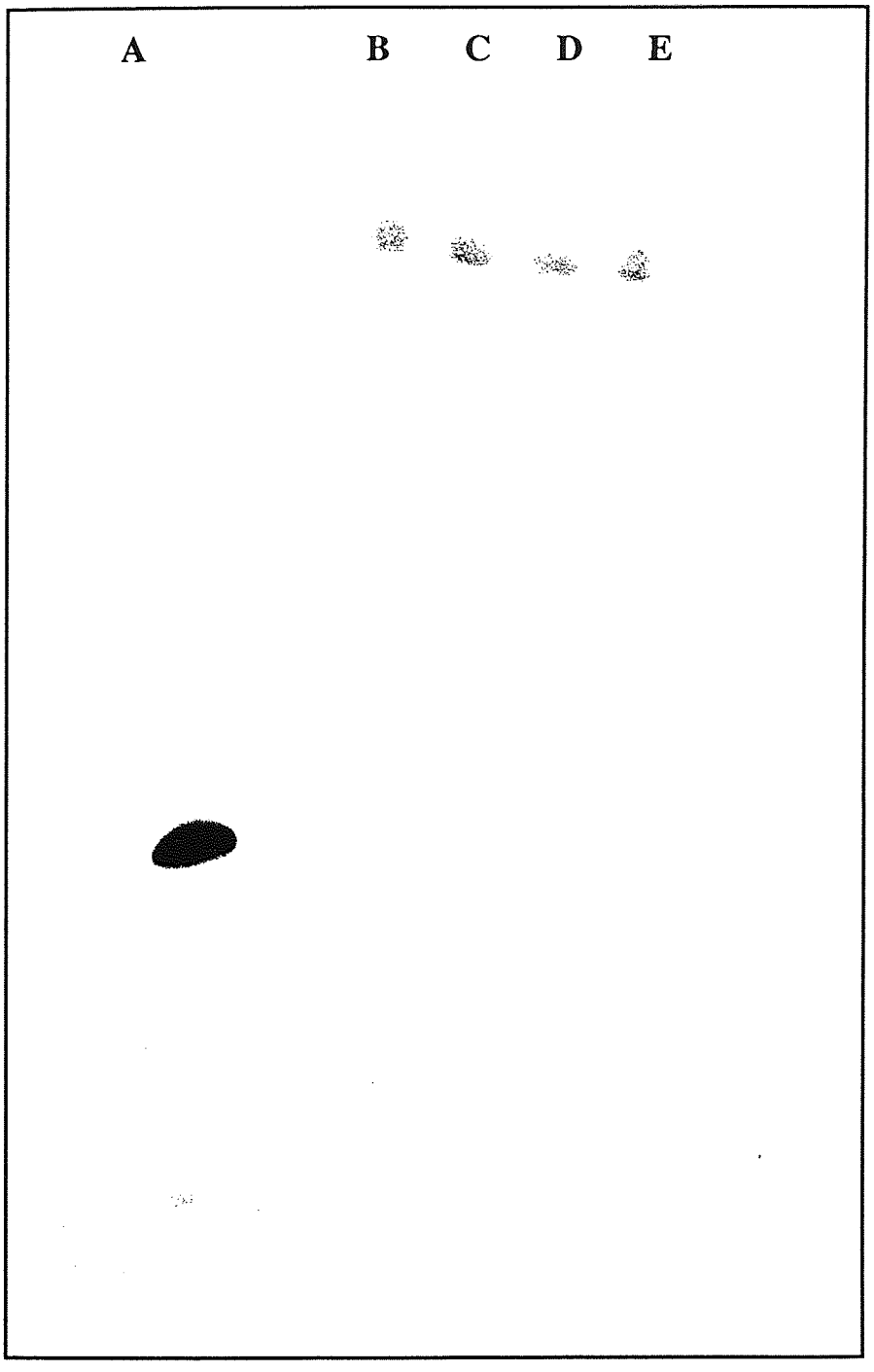
The monolayers were washed (1 x 500 µl x 10 min) at 37°C with M4 at pH 6, and then incubated at 37°C, with M4 containing 62.5 nM Gly-[^{3}H]L-Pro, 10 mM L-Pro and [^{32}P]-S-tat for 3 (C), 15 (D) and 18 min (E), with control-1 (A) being [γ^{32}P]-dATP, and control-2 (B) being [^{32}P]-S-tat in M4 with 10 mM L-Pro. Samples were taken from the wells after the appropriate time period and [^{32}P]-S-tat was assessed for binding and degradation by gel electrophoresis, to locate any free label ([γ^{32}P]-dATP) present.

FIGURE 7.8 *[^{32}P]-S-tat stability when incubated with Caco-2 cell monolayers*



The monolayers were washed (1 x 500 µl x 10 min) at 37°C with M4 at pH 6, and then incubated at 37°C, with M4 10 mM L-Pro and [^{32}P]-S-tat for 3 (C), 15 (D) and 18 min (E) on the cell sheet (1 x 250 µl), with control-1 (A) being [γ^{32}P]-dATP, and control-2 (B) being [^{32}P]-S-tat in M4 with 10 mM L-Pro. Samples were taken from the wells after the appropriate time period and [^{32}P]-S-tat was assessed for binding and degradation by gel electrophoresis, to locate any free label ([γ^{32}P]-dATP) present.

FIGURE 7.9 *[^{32}P]-S-rev stability when incubated with Caco-2 cell monolayers*



The monolayers were washed (1 x 500 µl x 10 min) at 37°C with M4 at pH 6, and then incubated at 37°C, M4 containing 10 mM L-Pro and [^{32}P]-S-rev for 3 (C), 15 (D) and 18 min (E) on the cell sheet (1 x 250 µl), with control-1 (A) being [γ^{32}P]-dATP, and control-2 (B) being [^{32}P]-S-rev in M4 with 10 mM L-Pro. Samples were taken from the wells after the appropriate time period and [^{32}P]-S-rev was assessed for binding and degradation by gel electrophoresis, to locate any free label ([γ^{32}P]-dATP) present.

7.4 CONCLUSION

The uptake of Gly-[^{3}H]L-Pro was not effected by ATP, indicating that mononucleosides do not interact with the DTS. Control studies show no ODN binding to Gly-[^{3}H]L-Pro and no degradation of the ODN during incubation on the cell sheet. The conjugated ODN VitE-S-rev, interacted, probably non-specifically, with the DTS, producing 36.91 ± 2.13 % inhibition of Gly-[^{3}H]L-Pro uptake at pH 6. The DHDG-S-rev had no significant effect at pH 6, however both ODN-conjugates produced considerably greater inhibition at pH 5 (77.33 ± 5.10 % for VitE-S-rev and 72.70 ± 9.06 % for DHDG-S-rev). The increase in inhibition at the lower pH is consistent with increased cellular association of ODN at pH 5 [Beck *et al.*, 1996]. SQ-29852 and VitE-S-rev when co-administered together produced the same inhibition as seen with SQ-29852 alone, indicating that VitE-S-rev competes for the same binding sites on the DTS as the ACE-inhibitor, or that SQ-29852 has a greater affinity for these sites.

VitE-S-rev was able to block nearly all of the [^{32}P]-S-rev associated with the Caco-2 cells, at pH 6. However, DHDG-S-rev was only able to inhibit 38.36 ± 3.31 % of cell association at pH 6, suggesting that surface-bound DHDG-S-rev occupies different sites to those of the unconjugated ODN.

In conclusion, lipophilic ODN-conjugates designed to improve cellular delivery can also exert non-specific and potentially adverse effects. VitE-S-rev and DHDG-S-rev, especially at the lower pH, can dramatically affect the functioning of the DTS in Caco-2 cells. This could potentially compromise the absorption of nutrient peptides and important drugs (*e.g.* cephalosporins) should ODN-conjugates be administered orally, or possibly effect systemic delivery by interfering with absorption profiles in non-target cells. This also indicates that effect of ODNs and their lipophilic conjugates on other active transport processes should be investigated.

CHAPTER EIGHT

CONCLUSION

ABSTRACT

The structural requirements needed for interaction of peptides, peptidomimetics and other drug candidates with the DTS in Caco-2 cell monolayers, are discussed in this chapter.

Several classes of drugs such as the ACE-inhibitors and β-lactam antibiotics, which have structural similarities to dipeptides, have been found to be substrates for the DTS [Hu & Amidon, 1988, 1989: Nakashima *et al.*, 1984a, b: Friedman & Amdion, 1989a, b: Dantzig & Bergin, 1990, 1992, 1994: Thwaites *et al.*, 1995: Nicklin *et al.*, 1996]. As a result, these compounds show a higher degree of oral bioavailability than anticipated from mere passive diffusion and, thus, achieve adequate oral absorption to promote a therapeutic effect. Information regarding the structural features that optimise or ensure interaction with the DTS will be applicable to industry, where the knowledge can be applied to drug discovery and development programs, to aid in the design or modification of potential drug substrates for the DTS. Currently, the structural requirements needed for interaction have yet to be clearly defined, as no systematic study has been carried out. Previous studies attempting to define the structural requirements used only a small number of commercially available competitors, which focused on stereochemical preferences and substitutions at the carboxyl and amide terminal functions. The studies used a large excess of competitor at concentrations from 10-20 mM. If the concentration greatly exceeds the K_i of the competitor for the DTS, the system will be saturated, preventing the affinity of the competitors from being ranked.

Previous studies have used either Gly-Sar [Thwaites *et al.*, 1993a, b, c, g, 1994a] or cephalosporins [Dantzig & Bergin, 1990, 1992, 1994: Gochoco *et al.*, 1994: Eddy *et al.*, 1995] as probe compounds, however, the present study which uses Gly-[^{3}H]L-Pro, has shown that the affinity of competitors which are substrates for the DTS can be ranked with regard to interaction; this was achieved by using a low competitor concentration (1 mM) in order to prevent saturation of the system. If inhibition was not achieved at the concentration tesetd the experiment was repeated using a higher concentration, supply and solubility permitting, and in a similar manner competitor concentration was decreased if inhibition was greater than 85 %. Enzyme expression in Caco-2 cells was found to be effected by location, illustrating that cells differed between laboratories. This is probably due to forced selection of various sub-clones brought about by differences in external factors such as incubator conditions and feeding regimes. The DTS characteristics (*e.g.* inhibition by SQ-29852) were effected by increasing passage numbers, indicating that expression and characteristics are dependent upon cell age.

Using analogues of the L-Lys-L-Pro type ACE-inhibitor, SQ-29852, structural features which optimise interaction were defined. A hydrophobic function in domain A was the preferred substrate, with the presence of a positive charge, H^+-acceptors and H^+-donors decreasing affinity for the transporter. SQ-29852 analogues were more tolerant to substituents in the C domain compared to Gly-L-Pro analogues. This suggests that there are other interactions occurring along the length of the SQ-29852 molecule that override the effect of the functional group in domain C. SQ-29852 analogues illustrated a preference for a positive charge (Chapter 4), but L-norLeu-L-Pro indicated that a neutral function is optimal

for interaction in this domain. However, L-norLeu-L-Pro had an additional primary amine function, and therefore results are not directly comparable.

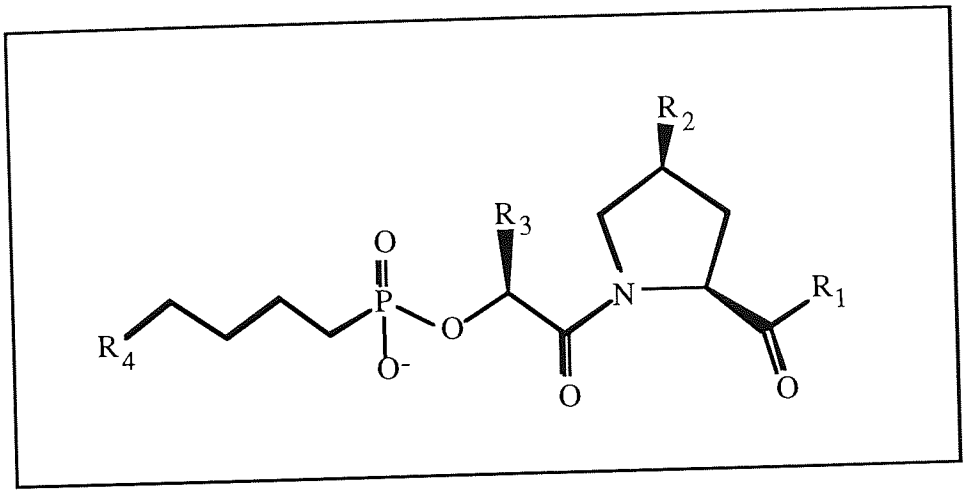
The most important finding, with respect to improving interaction by substituting with selected functions, was the fact the presence of a lipophilic group in domain D of the SQ-29852 analogues, *via* attachment to the proline ring at position 4 and attached to the seven-membered heterocyclic ring in the ACE-NEP inhibitors, significantly improved recognition with the transporter. Another important aspect of the work was the illustration that substitutions and the effect they have on interaction for one series of compounds does not hold true for all structures, highlighting the fact that the presence of multiple recognition sites on the molecule, may override the deleterious effect of any one change.

The DTS exhibited a preference for the L, L-enantiomers of all series of dipeptides tested. The D, D-enantiomer appeared to have the least affinity for the transporter, however for SQ-29852 and Lys-Pro enantiomers, the activity seen with D, D-enantiomer was the same as one of the mixed enantiomers, R, S (D, L) enantiomer for SQ-29852, and L-Lys-D-Pro. This suggests that D, D dipeptides, although exhibiting a lower affinity for the DTS, are still substrates, and illustrates that there are no general rules governing the interaction of the dipeptides containing mixed enantiomers, this being dependent upon amino acid composition.

From all of the information gained throughout this study, a hypothetical peptide structure based upon the SQ-2985 structure, has been proposed (Figure 8.1) and compared to the previously suggested structure [Kramer *et al.*, 1995]. R_1 and X correspond to the A domain, which in the present model is suggested to be a hydrophobic group, whereas Kramer *et al.* suggest that X should be a proton acceptor (*e.g.* thiol) with no indication being made as to the structure of R_1. R_3, which correspond to domain C must not be a negatively charged group [Kramer *et al.*, 1995], however interaction of 2-amino-adipyl-L-Pro illustrated that negatively charged groups have reduced activity but still show affinity for the DTS. Domain C is suggested to be a uncharged function for dipeptide analogues, but a positive charged function for ACE-inhibitor and ACE-inhibitor analogues. R_2 (domain D) is suggested to be a small neutral function (*e.g.* H, CH_3, vinyl), however, large lipophilic functions in this region dramatically increased interaction with the DTS in the present model. A free carboxyl group is present in the Kramer model, however, modification of this function for a SQ-29852 analogue (SQ-31065) increased affinity, but for Gly-L-Pro analogues had a negative effect. The discrepancy between the Kramer structure and the present one, may possibly be due to the fact that two different models and probes have been used, namely Caco-2 cells and Gly-[^{3}H]L-Pro compared to rabbit intestinal BBMV and cephalexin. Previously, the rabbit intestine has been shown not to be a comparable model to the human situation, and thus, may explain the apparent differences. The cephalosporins have a lower affinity for the DTS than the dipeptides (natural substrates) and they has also been some evidence to suggest that they are taken up *via* a separate system [Iseki *et al.*, 1989: Muranushi *et al.*, 1989: Sugawara *et al.*, 1991, 1994: Muranushi *et al.*, 1995:

Nicklin *et al.*, 1996]. The presence of a second system primarily for cephalosporins, but still retaining a certain degree of affinity for di- /tripeptides is not an unrealistic one. If two systems were present and Gly-L-Pro is a substrate for both, uptake into the cell would be the sum of uptake by both transporters. However, kinetic analysis provided no evidence for this, and therefore a second transporter system for which the probe compound is a substrate is unlikely.

FIGURE 8.1 *Proposed hypothetical peptide-like structure for optimum interaction with the DTS (2)*



R_1 = Negatively charged group, or in some instances an esterified function,

R_2 = Lipophilic function,

R_3 = Neutral charged function for dipeptide-like molecules, and a positively charged group (*e.g.* $^+NH_3$) for ACE-inhibitor analogues,

R_4 = Hydrophobic function.

Although, in the present system, structural features have been defined to increase interaction with the DTS, the mechanisms of interaction with the transporter site are not known, and this is an area requiring further study. However, molecular modelling of four of the ACE-NEP inhibitors revealed that the three carbonyl function can be oriented in a similar direction, which is in a local minimised energy state, suggesting that these may possibly be involved in hydrogen bond formation with the transporter site.

The relevance of the present system and current data are highlighted by a study investigating lisinopril and lisinopril analogue activity with ACE and oral bioavailability [Patchett, 1993]. The ornithine analogue of lisinopril ($\{CH_2\}_3NH_2$ in the C domain) was well absorbed in rats, however, the *N*-acetyl analogue (neutral function) displayed poor oral absorption, suggesting a basic function is required [Patchett, 1993]. The present data supports this, as a positive charge ($^+NH_3$) in the C domain appeared to be essential for interaction of SQ-29852 analogues, with reduced affinity being seen with an uncharged

function in this area (SQ-29907, C_4H_9 in the C domain). A guanadino function in the C domain of lisinopril resulted in poor oral absorption compared to the parent compound [Patchett, 1993]. A similar effect was seen with the current system, with guanadino analogues of SQ-29852, SQ-30403 and SQ-30472, both showing a lower affinity for the DTS. Thus illustrating that, in this instance, the present model is directly comparable to *in vivo* data, highlighting the usefulness of the present Caco-2 system.

All of the ACE-inhibitors tested displayed a degree of affinity for the DTS, however, previous data has shown that several compounds do not have carrier-mediated transport [Duchin *et al.*, 1982: Friedman & Amidon, 1989b: Swaan et al., 1995: Thwaites *et al.*, 1995: Morrison *et al.*, 1996]. The possibility that some of the ACE-inhibitors are simply binding the transporter site, and thus, not translocated into the cell, cannot be overlooked. A previous report found no relationship between affinity for the transporter and transepithelial transport [Eddy *et al.*, 1995]. However, in this instance cephalexin was used as the probe compound. This is a disadvantage of the present study that the system cannot differentiate between transported compounds and non-transported compounds and, therefore, a transport model would be appropriate. However, in defence of the uptake system, the Caco-2 cell model has previously been suggested to underexpress the DTS, with transport of SQ-29852 being considerably lower in the Caco-2 cell model than in the human situation [Chong *et al.*, 1996: Nicklin *et al.*, 1996]. Measuring transport of the analogues may possibly be underestimated and give a false result and, therefore, a system is needed that measures intracellular accumulation of a compound. In a similar sense, inhibition of the probe compound was seen with ODN-conjugates (VitE), which are not anticipated to be substrates for the transporter. ODN-conjuagte binding was increased with decreasing pH (pH 6-pH 5), due to a non-specific effects brought about by higher cell association resulting from possible protonation of the histidine residues at the transporter site, which is consistent with a previous study [Beck et al., 1996]. Therefore, non-specific actions of drugs forming oral dosing regimes which coincide with food consumption should be investigated to assess impact with the DTS, as it may have a nutritional impact.

The series of dipeptides tested had a higher degree of interaction with the transporter than the cephalosporins investigated. Previously, cephradine and cephalexin have been used on numerous occasions as probe compounds for the DTS [Eddy *et al.*, 1995: Hidalgo *et al.*, 1995]. The suitability of this class of drug as probe compounds is questioned for several reasons. Firstly, the lower affinity for the DTS, which can be explained by the structural relationship of the β-lactam antibiotics, to D-Ala-D-Ala. The present study, along with a number of other reports, has illustrated a stereochemical preference for the L-enantiomers [Asatoor *et al.*, 1973: Boyd & Ward, 1982: Matthews, 1987: Thwaites *et al.*, 1994a: Hidalgo *et al.*, 1995] and, therefore, it makes sense to use a compound with L, L-stereochemistry to maximise interaction of the probe. Secondly, previous studies have provided evidence to suggest that the β-lactam antibiotics are transported by a second transport system [Iseki *et al.*, 1989: Muranushi *et al.*, 1989: Sugawara *et al.*, 1991, 1994:

Muranushi *et al.*, 1995: Nicklin *et al.*, 1996]. Therefore, a dipeptide exhibiting a reasonable degree of stability, such as Gly-Sar or Gly-L-Pro are suggested as more appropriate probes than the cephalosporins.

The extension of this work in order to further define the DTS and its binding site is required. Future work may progress down one of six avenues:

- Molecular modelling of the conformationally constrained ACE-NEP inhibitors in order to learn more about the DTS binding sites and how molecules interact with them, possibly using the Catalyst program.

- Another possibility is to take the uptake system one step further and measure translocation of compounds into the cell *via* the DTS. A conventional transport model would be inappropriate due to the under expression of the DTS [Chong *et al.*, 1996: Nicklin *et al.*, 1996]. Using the BCECF system, where transport is correlated to pH intracellular changes due to dipeptide/H^+ cotransport, may be a possible alternative [Thwaites *et al.*, 1993a, b, c, g, 1994a].

- Another area of future work is the application of the present data (compound displaying the highest affinity within each domain group) to the design of an *in vivo* system to address the question, "does a lipophilic group in domain D which considerably improves affinity for the DTS in the Caco-2 model, have the same effect on oral bioavailability ?"

- Exploring the viability of applying the information gained to industry may possibly be achieved by selecting a poorly absorbed peptidomimetic drug and chemically modifying it to fit the proposed hypothetical structure, and assessing impact on its target (ACE for ACE-inhibitors) and oral bioavailability.

- Investigating the extent to which peptides and peptidomimetic compounds are transported out of the cell by the P-glycoprotein efflux system at the apical membrane, will provide a more accurate picture as to overall transport of a compound.

- Finally, studying the transport of a handful of prodrug conjugates which are recognised by the DTS, and comparing then with poorly absorbed drugs. Also studying the factors which allow uptake and transport out of the cell, and the release of the drug from the prodrug.

In conclusion, the present system represent a useful model when investigating structural requirements for the DTS, and highlights the possibility of improving current substrates by structural and stereochemical modification.

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APPENDIX ONE

ABBREVIATIONS

mAb	Monoclonal antibody
ABL	Aqueous boundary layer
ACE	Angiotensin-converting enzyme
ACE-NEP	Angiotensin-converting enzyme-neutral endopeptidase
AIDS	Acquired iummodeficiency syndrome
Ag I	Angiotensin I
Ag II	Angiotensin II
2-amino adipyl-L-Pro	2-amino adipyl-L-proline
β-Ala	β-alanine
D-Ala	D-alanine
DL-Ala	DL-alanine
L-Ala	L-alanine
D-Ala-D-Ala	D-alanyl-D-alanine
DL-Ala-DL-Ala	DL-alanyl-DL-alanine
L-Ala-L-Ala	L-alanyl-L-alanine
β-Ala-L-His	β-alanyl-L-histidine (carnosine)
L-Ala-L-Glu	L-alanyl-L-glutamate
L-Ala-Gly	L-alanylglycine
β-Ala-Gly-Gly	β-alanylglycylglycine
D-Ala-D-Phe	D-alanyl-D-phenylalanine
D-Ala-L-Phe	D-alanyl-L-phenylalanine
L-Ala-D-Phe	L-alanyl-D-phenylalanine
D-Ala-D-Pro	D-alanyl-D-proline
D-Ala-L-Pro	D-alanyl-L-proline
L-Ala-D-Pro	L-alanyl-D-proline
L-Ala-L-Pro	L-alanyl-L-proline
L-Ala-L-Val	L-alanyl-L-valine
ANP	Atrial natriuretic peptide
L-Arg	L-arginine
D-Asp	D-aspartic acid
L-Asp	L-aspartic acid
L-Asp-L-Asp	L-aspartyl-L-aspartate
L-Asp-L-Lys	L-aspartyl-L-lysine
L-Asp-D-Phe	L-aspartyl-D-phenylalanine
L-Asp-D-Pro	L-aspartyl-D-proline
ATP	Adenosine triphosphate
ATTC	American Type Tissue Culture Collection
BBMV	Brush border membrane vesicle
BCA	Bicinchoninic acid
BCECF	2', 7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein

N-Boc	*N*-tert-butoxy-carbonyl
°C	Degrees Celsius
Cbl	Cyanocobalamin (vitamin B 12)
Ci	Curie
CHO	Chinese hamster ovary
CH_3-18	[3S, 4S]-4-[*N*-morpholinoacetyl-(1-naphthyl)-L-alanyl-*N*-methyl-[4-pyridyl]-1-pentanone
cm	Centimetre
CO_2	Carbon dioxide
CPM	Counts per minute
Cu	Copper
Cys	Cysteine
d	Day
Da	Dalton
kDa	Kilodalton
DEPC	Diethylpyrocarbonate
DHDG	2-Di-*O*-hexadecyl-3-glycerol
DIDS	4, 4'-Diisothiocyanatostilbene-2, 2'-disulphonic acid
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
cDNA	Complementary deoxyribonucleic acid
DNP	2, 4-dinitrophenol
L-Dopa	L-3, 4-dihydroxyphenylalanine
$D_{o/b}$	Octanol/buffer distributuion coefficient of drugs
DPM	Decays per minute
DTS	Dipeptide transport system
E_a	Activation energy
ECACC	European Collection of Animal Cell Culture
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FBS	Foetal bovine serum
FCCP	Carbonylcyanide *p*-trifluoromethoxyphenylhydrazone
g	Gram
GABA	γ-amino-*n*-butyric acid
GALT	Gut-associated lymphoid tissue
GI	Gastrointestinal
L-Glu	L-glutamic acid
L-Glu-L-Ala-L-Ala	L-glutamyl-L-alanyl-L-alanine
pGlu-L-Dopa-L-Pro	pglutamyl-L-dihydroxyphenylalanyl-L-proline

L-Glu-L-Glu	L-glutamyl-L-glutamate
GLUT	Glucose transporter protein
Gly	Glycine
Gly-L-Ala	Glycyl-L-alanine
Gly-Gly	Glycylglycine
Gly-L-Glu	Glycylglutamate
Gly-Gly-Gly	Glycylglycylglycine
Gly-Gly-L-Phe	Glycylglycine-L-phenylalanine
Gly-L-Leu	Glycyl-L-leucine
Gly-DL-Met	Glycyl-DL-methionine
Gly-DL-Phe	Glycyl-DL-phenylalanine
Gly-L-Phe	Glycyl-L-phenylalanine
Gly-[^{3}H]L-Pro	Glycyl-3, 4-[^{3}H]L-proline
Gly-L-Pro	Glycyl-L-proline
Gly-L-Pro-L-Arg	Glycyl-L-prolyl-L-arginine
Gly-L-Pro-Gly-Gly	Glycyl-L-prolylglycylgylcine
Gly-Sar	Glycyl-sarcosine
Gly-Sar-Sar	Glycylsarcosylsarcosine
Gly-Sar-Sar-Sar	Glycylsarcosylsarcosylsarcosine
Gly-L-Trp	Glycyl-L-trptophan
Gly-L-Tyr	Glycyl-L-tyrosine
h	Hour
HBSS	Hank's balanced salt solution
He	Helium
HEPES	*N*-[2-hydroxyethyl]piperazine-*N'*-]2-ethansulphonic acid
$HgCl_2$	Mercury (II) chloride
L-His	L-histidine
L-His-L-Ala	L-histidyl-L-alanine
L-His-Gly	L-histidylglycine
HIV	Human immunodeficiency virus
hpt-1	cDNA encoding for Caco-2 peptide transporter protein
hPEPT1	cDNA encoding for human intestinal peptide transport protein
HPLC	High-performance liquid chromatography
H_3PO_4	Orthophosphoric acid
I	Competitor concentration
IC_{50}	Concentration of inhibitor that causes 50 % inhibition of uptake
IF	Intrinsic factor
IgA	Immunoglobulin A

IgG	Immunoglobulin G
J_{max}	Maximal velocity
k_d	Diffusional rate constant
Kg	Kilogram
K_i	Inhibition constant
kJ	Kilojoule
K_m	Michaelis constant
K_t	Michaelis constant
l	Litre
LDL	Low density lipoprotein
L-Leu	L-leucine
L-Leu-L-Ala	L-leucyl-L-alanine
D-Leu-D-Leu	D-leucyl-D-leucine
L-Leu-L-Leu	L-leucyl-L-leucine
L-Leu-L-Val	L-leucyl-L-valine
LNAA	Large neutral amino acid
Log P	Logarithm of the partition coefficient
LSC	Liquid scintillation counting
D-Lys	D-lysine
L-Lys	L-lysine
D-Lys-D-Pro	D-lysyl-D-proline
D-Lys-L-Pro	D-lysyl-L-proline
L-Lys-D-Pro	L-lysyl-D-proline
L-Lys-L-Pro	L-lysyl-L-proline
M	Molar
m	metre
MAP	Metabolism and Pharmacokinetic
mCi	Millicurie
MDP	Membrane dipeptidase
MDR	Multidrug resistance
MES	2-[*N*-morpholino]ethanosulfonic acid
L-Met	L-methionine
L-Met-L-Met	L-methionyl-L-methionine
min	Minute
mg	Milligram
ml	Millilitre
mm	Millimetre
mM	Millimolar
mmol	Millimole
mol	Mole

MP	Methylphosphonate
NaCl	Sodium chloride
NaN_3	Sodium azide
NaOH	Sodium hydroxide
NEAA	Non-essential amino acids
NEP	Neutral endopeptidase
NEN	New England Nuclear
ng	Nanogram
nM	Nanomolar
nm	Nanometre
nmol	Nanomole
L-norLeu	L-norLeucine
L-norLeu-L-Pro	L-norleucyl-L-proline
ODN	Oligodeoxynucleotide
ODS	Octadecyl silica
ODS-2	Octadecyl silane-2
L-Orn	L-ornithine
L-Orn-L-Pro	L-ornithyl-L-proline
P_{app}	Apparent permeability coefficient
PBS	Phosphate buffered saline
PBS-azide	Phosphate buffererd saline-sodium azide
PCNBS	p-cholromercuibenzene
PDBu	Phorbol 12, 13-dibutyrate
PDD	4a-phorbol, 12, 13 didecanoate
PEG	Polyethylene glycol
PepT1	Protein associated with the rabbit intestinal dipeptide transport system
PepT2	Protein associated with the human renal dipeptide transport system
D-Phe	D-phenylalanine
L-Phe	L-phenylalanine
D-Phe-D-Ala	D-phenylalanyl-D-alanine
D-Phe-L-Ala	D-phenylalanyl-L-alanine
L-Phe-L-Ala	L-phenylalanyl-L-alanine
L-Phe-D-Ala	L-phenylalanyl-D-alanine
L-Phe-L-Ala-L-Ala	L-phenylalanyl-L-alanyl-L-alanine
L-Phe-L-Ala-L-Pro	L-phenylalanyl-L-alanyl-L-proline
D-Phe-Gly	D-phenylalanylglycine
L-Phe-Gly	L-phenylalanylglycine
L-Phe-Gly-Gly	L-phenylalanylglycylgylcine

L-Phe-L-Pro	L-phenylalanyl-L-proline
pH_i	Intracellular pH
P_i	Inorganic phosphate
pmol	Picomole
PMA	Phorbol 12-myristate 13-acetate
PNA	Peptide-nucleic acid analogue
PO	Phosphodiester
PS	Phosphorothioate
[^{3}H]L-Pro	L-[2, 3, 4, 5-^{3}H]proline
L-Pro	L-proline
L-Pro-Gly	L-prolylglycine
L-Pro-L-Phe	L-prolyl-L-phenylalanine
R	Universal gas constant
r	Ratio of the uptake rate in the absence of a competitor, to the value in the presence of a competitor
RAS	Renin-angiotensin-system
rat PepT1	cDNA for rat intestinal peptide transporter protein
RF	Riboflavine
RNA	Ribose nucleic acid
mRNA	Messenger ribose nucleic acid
RNase H	Ribonuclease H
S	Substrate concentration
Sar	Sarcosine
Sar-L-Pro	Sarcosyl-L-proline
S.D.	Standard deviation
sec	Second
L-Ser	L-serine
S_0	Initial substrate concentration
S_t	Substrate concentration at time t
T	Temperature [Kelvin]
T_4	Tetraiodoothyronine
TA	Taurocholic acid
TCII	Transcobalamin II
TER	Transepithelial electrical resistance
TFA	Trifluoroacetic acid
TLC	Thin-layer chromatography
TRH	Thyrotrophin-releasing hormone
L-Trp	L-tryptophan
L-Trp-Gly	L-tryptophylglycine
L-Tyr	L-tyrosine

L-Tyr-Gly	L-tyrosylglycine
TX100	Triton X100
U	Units
UWL	Unstirred water layer
L-Val	L-valine
L-Val-L-Ser	L-valyl-L-serine
D-Val-D-Val	D-valyl-D-valine
D-Val-L-Val	D-valyl-L-valine
L-Val-D-Val	L-valyl-D-valine
L-Val-L-Val	L-valyl-L-valine
VitE	Vitamin E
V_{max}	Maximal velocity
V_a	Uptake in the absence of a competitor
V_o	Rate of uptake/transport
V_i	Uptake in the presence of a competitor
x	% inhibition value $\geq$ 100.00
y	% inhibition $\leq$ 0.00
%	Percent
µg	Microgram
µl	Microlitre
µm	Micrometre
µM	Micromolar
µmol	Micromole
Ω	Ohm

APPENDIX TWO

IC_{50} CALCULATION

EXAMPLE OF A SAMPLE CALCULATION, FROM DPM TO IC_{50}

IC_{50} values are calculated using four steps;

1) The specific activity of the radiolabelled compound is worked out experimentally from the stock dpm:

50 nM Gly-[^{3}H]L-Pro in a 250 µl volume was used, therefore:

$$50 \text{ nM} = 50 \text{ nmol } l^{-1} = 50 \text{ pmol } ml^{-1}$$

$$50 \times 0.25 = 12.5 \text{ pmole}$$

$$\text{Specific activity} = \frac{\text{Stock dpm}}{\text{amount used}} = \frac{1754792 \text{ dpm}}{12.5 \text{ pmole}} = 140383.4 \text{ dpm pmole}^{-1}$$

2) The amount of radiolabelled compound in the cells is calculated;

$$\text{Amount in cells} = \frac{\text{dpm of cell suspension}}{\text{Specific activity}}$$

$$\text{Amount in cells} = \frac{47648.6 \text{ dpm}}{140383.4 \text{ dpm pmole}^{-1}} = 0.340 \text{ pmole}$$

3) Correct for 15 % non-specific (passive) uptake into cells (section 3.3.1);

$$\text{Non-specific uptake} = \frac{0.340 \text{ pmole}}{100} \times 15 = 0.051 \text{ pmole}$$

$$\text{Amount in cells (specific uptake)} = 0.340 \text{ pmole} - 0.051 \text{ pmole} = 0.289 \text{ pmole}$$

4) IC_{50} calculation:

$$IC_{50} = \frac{[I]}{r-1}$$

where,

$$r = \frac{V_a}{V_i}$$

with [I] being the competitor concentration, V_a being uptake in the absence of competitor (control), and V_i being uptake in the presence of competitor.

EXAMPLE:

Control = 0.283 pmole in cells, uptake of probe in presence of 1 mM SQ-29852 = 0.111 pmole, therefore:

$$r = \frac{V_a}{V_i} = \frac{0.283}{0.111} = 2.550$$

$$IC_{50} = \frac{1}{2.550 - 1} = 0.645 \text{ mM}$$

SQ-29852 has an IC_{50} value of 0.645 mM in this instance.