INVESTIGATING THE MODULATION OF ORAL DRUG ABSORPTION USING *IN VITRO* MODELS

JONATHAN ROBERT BIGGS

Doctor of Philosophy

ASTON UNIVERSITY

JULY 2003

ASTON UNIVERSITY

INVESTIGATING THE MODULATION OF ORAL DRUG ABSORPTION USING *IN VITRO* MODELS

JONATHAN ROBERT BIGGS

Doctor of Philosophy

JULY 2003

SUMMARY

Dipeptides can be absorbed into cells *via* the dipeptide transporter (which also transported tripeptides and dipeptide derivatives). The optimum conditions for measuring the inhibition of Gly-Pro uptake in Caco-2 cells were identified. A number of structure-activity relationships were identified. These included the effects of increasing the amino-acid chain-length, and the presence of a thiol or hydroxyl group in the side-chain increased IC_{50} while the presence of a hydroxyl group did not. The benzyl esters had lower or equal IC_{50} values compared to the parent dipeptides while the methyl esters had higher values. These results indicated that while molecular properties did affect IC_{50}, the size, charge and composition of three particular groups caused the most significant effects, supporting the structure-activity relationships identified.

An assay was developed using calcein-AM to show the inhibition of p-glycoprotein activity. There was no significant change due to the presence of mannitol but there was in the presence of cyclosporin A ($p<0.01$). Incubating the cells with the test solution for 30 minutes before the addition of the ester resulted in a significant ($p<0.001$) difference. The assay was specific for p-glycoprotein, as the presence MRP inhibitors had no effect ($p>0.05$). The modified protocol allowed the identification of p-glycoprotein inhibitors quickly and simply using a cell suspension of unmodified cells.

The clinically relevant buffering of grapefruit juice to pH 7 led to a four-fold increase in intracellular calcein and hence significant inhibition of p-glycoprotein. Buffered orange and lemon juices had no effect on the assay. Flavone derivatives had previously been found to be inhibitors of CYP3A4 yet neither naringin nor naringenin had any significant effect at concentrations found in grapefruit juice. Of the other (non-grapefruit) flavone derivatives tested, hesperidin, found in orange juice, had no significant effect, kaempferol and rutin also had no effect while genistein significantly inhibited p-glycoprotein (results that support previous studies). Hydroxycinnamic acids had no effect on p-glycoprotein. Studies on other compounds found that the balance between inhibiting p-glycoprotein and disrupting cell membranes depends on the compound containing an oxygen atom and the size of the negative charge on it, as well as three-dimensional arrangement of the atoms.

Key words: Caco-2, dipeptide transporter, glycyl-L-proline, calcein-AM, p-glycoprotein (p-gp), cyclosporin A, MRP, grapefruit juice, natural products.

DEDICATION

I would like to dedicate this thesis to the memory of my grandmother, Hilde Biggs (Bomi) who was always interested in my work and always asking how my bugs were doing. I will miss her.

ACKNOWLEDGEMENTS

A Total technology case award from the Engineering and Physical Sciences Research Council (EPSRC) and Bristol-Myers Squibb jointly funded the work in this thesis. Thanks also to the Aston Business School for their help in attaining the MBA modules required.

I would like to thank my supervisor Professor W.J. Irwin for his help, guidance and patience throughout my time at Aston. I would also like to thank Professor P. Timmins for his support and encouragement.

I am grateful to the members the Pharmaceutics research group who have helped technically or emotionally with this work. Specifically I want to thank Chris Bache for keeping everything working, Christine Tran for trying to teach me everything she knew and Amelia Petch and Emma Bradbury for being there whenever I needed to complain.

Finally I would like to thank all of my family (especially my grandparents, parents and sister), friends and housemates for their constant encouragement, support and tolerance.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF EQUATIONS

ABBREVIATIONS

ABC	ATP-binding cassette
ACE	Angiotensin-converting enzyme
ACV	Acyclovir
ADP	Adenosine diphosphate
Ala	Alanine
AMBA	4-aminomethylbenzoic acid
ANOVA	Analysis of variance
APAA	4-aminophenylacetic acid
Arg	Arginine
Asp	Aspartic acid
ATP	Adenoside trisphosphate
AUC	Area under the curve
BBMV	Brush-border membrane vesicles
BCA	Bicinchoninic acid
cDNA	Cloned DNA
CHO	Chinese Hamster Ovary
C_{max}	Maximum concentration
CMV	Cytomegalovirus
cpm	Counts per minute
CsA	cyclosporin A
CYP	Cytochrome P450
Cys	Cysteine
Da / kDa	Daltons / Kilodaltons
Dip	Dipole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
dpm	Disintegrations per minute
EDTA	Ethylene-diamine-tetra-acetic acid
Glu	Glutamic acid
Gly	Glycine
HBSS	Hank's Balanced Salt Solution

HEPES	N-2 hydroxyproperazine–N'-2 ethanosulphonic acid
His	Histidine
HIV	Human immunodeficiency virus
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HoF	Heat of Formation
HOMO	Highest Occupied Molecular Orbit
IC50	Concentration that inhibits 50% activity
Km	Michaelis constant
Leu	Leucine
LUMO	Lowest Unoccupied Molecular Orbit
Lys	Lysine
MAO	Mono-amine oxidase
MDR	Multi-drug resistance
MES	2-[N-morpholino]ethanesulphonic acid
MOP	Methoxypsoralen
MR	Molar refractivity
mRNA	Messenger RNA
MRP	Multi-drug resistance protein
MSA	Molecular surface area
NADPH	Nicotinamide adenine diphosphate
NNK	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NRTI	Nucleoside reverse transcriptase inhibitors
Nva	Norvaline
OATP	Organic anion transport protein
Papp	Apparent permeability
PBS	Phosphate-buffered-saline
PGP	p-glycoprotein
Phe	Phenylalanine
pKa	Log of the ionisation constant
Pro	Proline
PUVA	Psoralen and UVA therapy
Sar	Sarcosine

t_{max}	Time to maximum concentration
Trp	Tryptophan
Val	Valine
Vmax	Maximum rate of uptake/transport

1. INTRODUCTION

ABSTRACT:

The general introduction gives an overview into oral drug absorption, focusing on active uptake transporters, and barriers to absorption, focusing on metabolic enzymes and efflux transporters. The Caco-2 cell line is reviewed as a model for these processes. Finally, the effect of natural compounds on the pharmacokinetics of co-administered drugs is also reviewed. The resulting aim of the thesis is to identify methods to increase drug absorption from the intestine into the blood.

1.1 The oral delivery of drugs

The oral route is the most common and convenient approach to drug delivery. This route has a number of barriers that drugs must pass before they are absorbed into the body. The barriers include the physical anatomy of the gastrointestinal tract and physiological and physiochemical factors such as the effect of pH. The different barriers are examined in detail below.

1.1.1 The small intestine

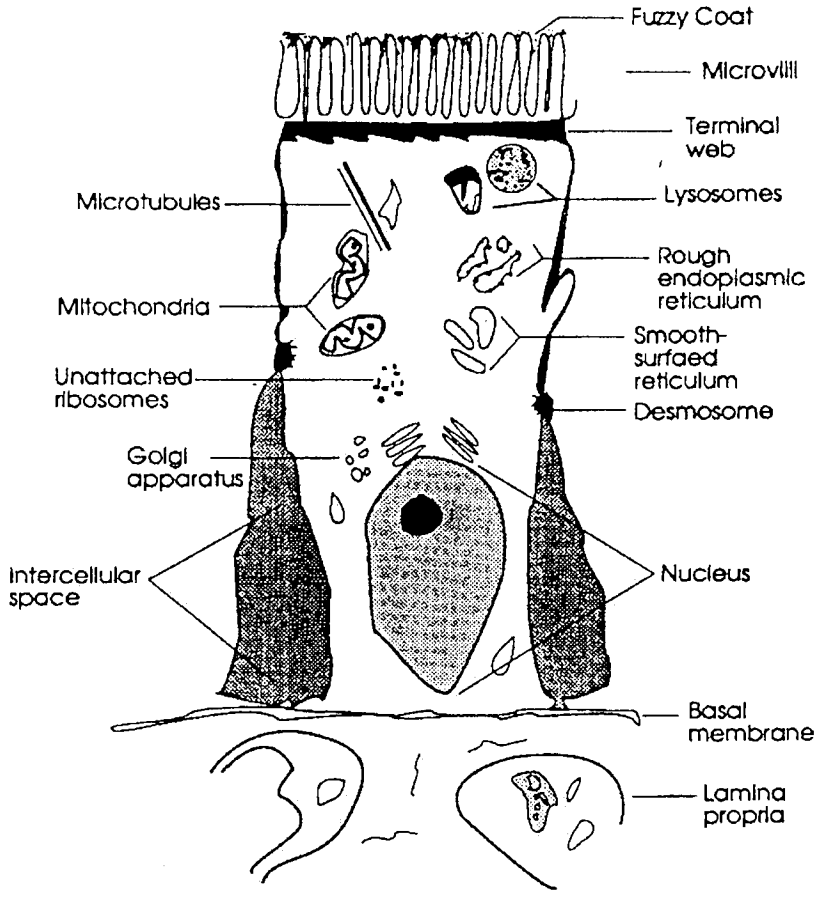
After ingestion, drugs mix with and dissolve into the acidic contents of the stomach but, due in part to the surface area and contact time, there is relatively little absorption from this organ (although small amounts of certain lipid-soluble compounds can be taken up, including aspirin, other non-steroidal anti-inflammatory drugs, and ethanol). Gastric emptying passes the contents of the stomach into the small intestine, which is the major site of food and drug absorption.

The small intestine is divided into three sections: duodenum (0.2 m), jejunum, and ileum (2.5 m together). The duodenum is where bile and pancreatic juices are secreted. There are no macroscopic differences between the jejunum and ileum although the ileum has more lymph nodules, known as Peyer's patches. The total surface area in humans is approximately 200 m^2 due to the presence of villi and microvilli. Villi are folds in the surface of the intestine (mucosa), about 1 mm long. Microvilli are folds in the apical surface of the intestinal cells. Closer examinations of the cells that make up the villi show that 90% are the same type: enterocytes [Kararli, 1989].

1.1.2 The structure and function of small intestine enterocytes

Enterocytes form the most important anatomical barrier against drug absorption. The cells are produced through the mitotic division and maturation of cells called Crypts of Lieberkuhn, located at the bottom of the villi. The maturing enterocytes continuously migrate up to the tips of the villi, where they are finally sloughed off into the intestine. The average life of these cells is three days [Kararli, 1989]. Figure 1.1 below is a representation of an enterocyte. The most distinctive feature of the cell is its apical brush-border membrane, composed of tightly packed microvilli.

Figure 1.1 Diagram of an enterocyte



Adapted from Figure 4, Kararli, 1989

The microvilli are folds in the surface of the cell and are approximately 1 μm long. On the outside of the cells, next to the microvilli, is the glycocalyx or "fuzzy coat" which is approximately 0.1 μm thick [Ito, 1974]. This coat is composed of glycoproteins anchored to the enterocyte membrane. The apical membrane, with its fuzzy coat, contains digestive enzymes such as sucrase, oligoaminopeptidases, maltase, and alkaline phosphatase [Kararli, 1989].

The core of a microvillus consists of parallel filaments of actin. These filaments are anchored to the cell membrane and connected to the network of filaments that form the terminal web [Mooseker & Tilney, 1975]. The terminal web extends across the apical cytoplasm just beneath the microvilli. The filaments of the terminal web are involved in the formation of junctional complexes, tight junctions (zonula occuladens), zonula adherens, and desmosomes (macula adherens). Tight junctions circle the apical end of

each epithelial cell and separate the contents of the intestinal lumen from intracellular spaces. They separate the apical and basolateral membranes. The zonula adherens and the desmosomes provide mechanical stability to the epithelium.

The presence of villi and microvilli creates a large surface area on the cells. The terminal web, composed of filaments of actin inside the cells, forms a number of junctional complexes with other cells to create an apical and basolateral surface of the cell monolayer.

1.1.3 The cellular composition of the small intestinal epithelium

Enterocytes are not the only cells found in the small intestine. Other cells include goblet cells, Paneth's cells, enteroendocrine cells, migrating leukocytes and M cells [Leeson *et al.*, 1988]. Goblet cells are scattered among the enterocytes in the villi and have a similar lifespan. They excrete an acidic glycoprotein that forms a protective layer on the glycocalyx (on the surface of the microvilli of the enterocytes). Paneth's cells are only found in the bases of the crypts (at the bottom of the villi). These cells have a slow renewal rate of thirty days and secrete lysozyme and other proteins. Lysozyme digests bacterial cell walls, possibly regulating intestinal microflora. Enteroendocrine cells are found in the crypts and on the villi. They secrete peptides that regulate gastric secretion, intestinal motility, pancreatic secretion, and gall-bladder contraction. Finally, M cells are found in the epithelium above Peyer's patches. M cells are specialised cells whose basolateral surface is closely related to lymphocytes lying in the epithelium. M cells transport macromolecules from the lumen of the intestine to these lymphocytes, where responses to foreign antigens can be undertaken.

1.1.4 The large intestine

After passing through the small intestine, the remaining food and drugs enter the colon, in the form of chyme (a thick semifluid mass of partly digested food). The colon has a length of 1.1 m and the surface does not have villi. Colonic enterocytes are slightly different to those in the small intestine, *i.e.* the microvilli are less closely packed and the fuzzy coat is less compact. The colon plays a major role in the absorption of water, sodium and other minerals. In the colon, there are hundreds of species of microorganisms, most of which are

anaerobic and are involved in reductive reactions. The colon converts chyme into faeces, which is then excreted from the body [Kararli, 1989].

1.2 Absorption mechanisms

There are two main methods of drug absorption into cells: paracellular (between the cells) and transcellular (through the cells). Transcellular transport can be divided into three mechanisms: passive, active carrier-mediated, and endocytotic. These mechanisms are examined below.

1.2.1 Paracellular transport

By definition, paracellular diffusion is when the drug passes between the tight junctions. Permeability is usually small compared to drugs that are transported passively or transcellularly. For example, mannitol's (paracellular) permeability is 150 times less than metoprolol's (transcellular) permeability. There is a size limit on the molecules that can fit between the junctions. Molecules with a molecular weight less than 200 Da are transported but at low rates. Significant paracellular transport only occurs with very small ions (*e.g.* Na^+ and Cl^-) and small molecules (*e.g.* urea). This route is sensitive to the effect of chelating agents that disrupt the tight junctions (by binding with calcium or magnesium ions).

1.2.2 Transcellular transport

1.2.2.1 Passive transport of drugs

Passive transport refers to the movement of a solute along a concentration or electrical gradient where the solute does not interact with the structural elements of the membrane. The rate is directly proportional to the gradient, is not saturable and cannot be inhibited by the presence of other drugs. The rate of movement of a drug across a membrane is proportional to the diffusion coefficient for the drug (related to the size of the molecule), the partition coefficient of the drug (a measure of solubility is logP defined as the logarithm of the partition coefficient of a compound between octanol and water), the surface area of the membrane, the thickness of the membrane, and the size of the concentration gradient across the membrane. These factors were used to derive the Noyes-Whitney equation, shown in Equation 1.1 below.

Equation 1.1 The Noyes-Whitney equation

$$\frac{dQ}{dt} = \frac{D \times A \times K \times (C_O - C_I)}{h}$$

Where:

$\frac{dQ}{dt}$ = The change in the number of drug molecules inside the membrane over time

D = The diffusion coefficient of the drug

A = The surface area of the membrane

K = The partition coefficient of the drug

$(C_O - C_I)$ = The concentration of the drug outside the membrane minus the concentration inside

h = The thickness of the membrane

Most unionised molecules can easily cross the plasma membrane due to their large partition coefficient. Many drugs are weak acids or bases. The rate of permeation of these compounds will depend on the concentration gradient of the unionised form.

The Henderson-Hasselbach equation (Equation 1.2 below) defines the relationship between pH, the ionisation constant for the drug (pKa) and the ratio of the ionised and unionised forms of the drug.

Equation 1.2 The Henderson-Hasselbach Equation for a weak acid*

$$pH = pKa + \log \frac{[ionized]}{[unionized]}$$

*for weak bases, the ratio is [unionised]/[ionised]

It follows that there is an interrelationship between the rate of drug absorption, the pKa of the diffusing drug, and the pH at the site of absorption. The pH at any site will affect the fraction of the drug that is unionised and so the rate of absorption. This relationship is known as the pH-partition theory. Although useful, calculated absorption rate constants show that this theory is too simple. The effects of the microclimate pH, unstirred water layer, and permeability of the membrane to the unionised form, all cause deviations from the theorised rates [Kararli, 1989].

Carrier-mediated transport can be either facilitated or active. Facilitated transport differs from passive transport as the solutes interact with membrane proteins. These proteins are capable of reversibly binding specific substrates. The substrates move along a concentration gradient so no energy source is needed. Very few energy free transporters have been identified, but glucose transport is thought to be a facilitated process rather than an active process [Karali, 1989].

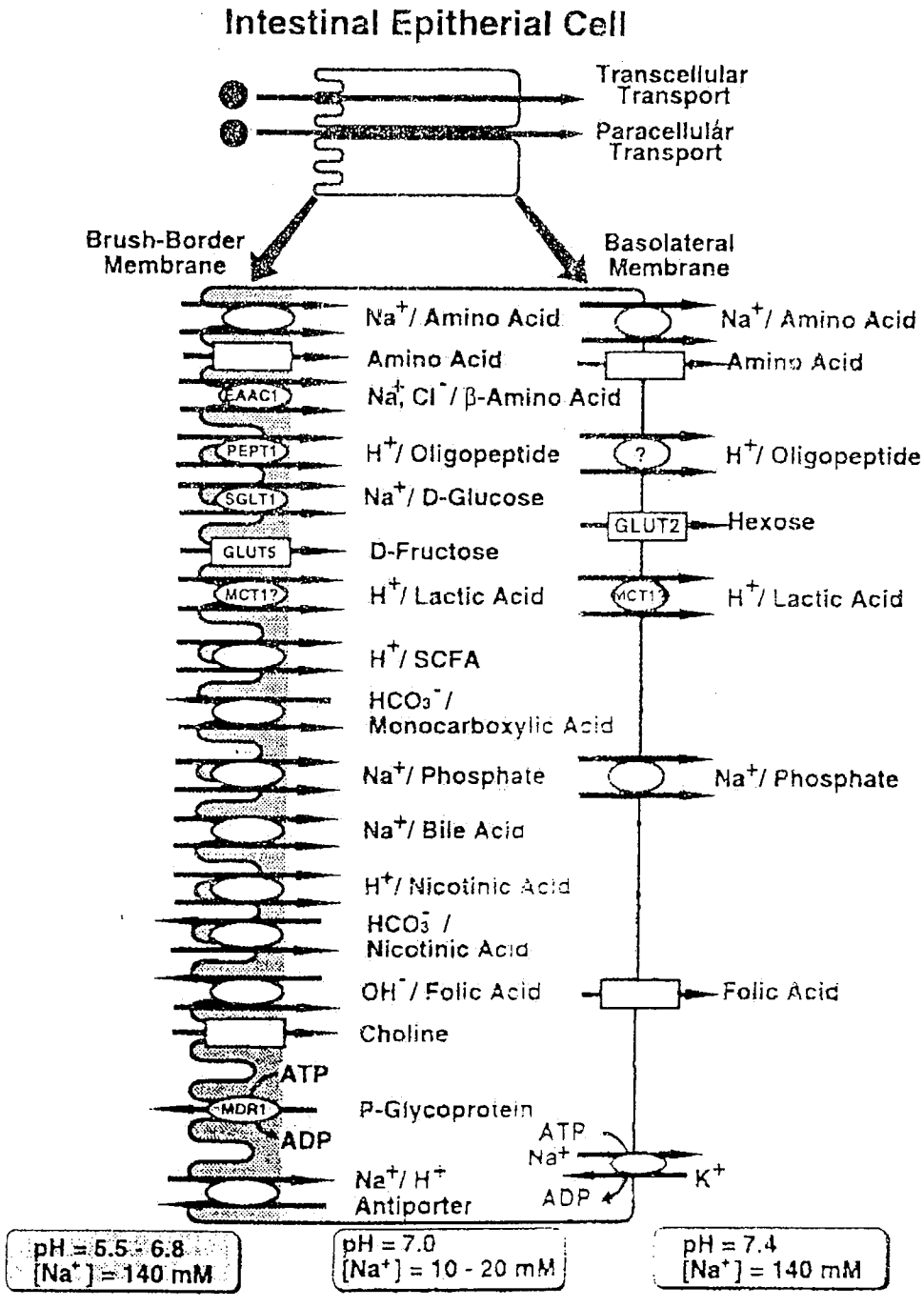
1.2.2.2 Active carrier-mediated transport of drugs

Active transport is the movement of a molecule across a membrane or another barrier that is driven by energy other than stored in the concentration gradient or the electrochemical gradient of the transported molecule. It can only occur at intact, closed membranes. These membranes can envelop very different compartments, for example the whole cell, vesicles, or the vacuole. As a result of active transport, ions and metabolites can be concentrated within the respective compartment or the cell and the steady state of metabolism can be kept constant despite large fluctuations in the composition of the external medium.

Active transport is similar to facilitated transport but it requires an energy source, which can be supplied in two ways. The first involves the hydrolysis of adenosine triphosphate (ATP). These transporters are known as pumps, *e.g.* Na^+/K^+ ATPase pump. Studies have shown that the electrochemical gradients of Na^+ and H^+ play a major role in the absorption of many compounds. These ions both have inwardly directed concentration gradients maintained by pumps. The second class of transporters uses these gradients to transport other compounds. These transporters are co-transporters, transporting both at the same times, *e.g.* Na^+/glucose transporter [Hopfer *et al.*, 1973]. In the sodium-glucose cotransport system, energy for the transport is provided by the high sodium ion concentration outside the cell compared to inside. The sodium ion concentration never comes to equilibrium inside and outside the cell because the sodium ions are pumped back out as they enter the cell. The sodium-glucose cotransport system is thus an active transport system that derives its energy from another active transport system-the sodium ion gradient maintained by the sodium-potassium pump. The transport of compounds through the transporters follows Michaelis-Menton kinetics (see Appendix A1). This means that other compounds can compete with the substrate to get transported and inhibit the transport of the substrate.

Figure 1.2 below (adapted from Tsuji & Tamai, 1996) is a summary of the range of intestinal epithelial transporters currently identified.

Figure 1.2 Summary of intestinal epithelial transporters



Adapted from Fig.1, Tsuji & Tamai, 1996.

1.2.3 Other transport mechanisms: Endocytosis & Receptor-mediated transporters

Endocytosis occurs when a small portion of the plasma membrane progressively encloses materials near the surface of the cell. The membrane first invaginates and then pinches off to form an intracellular vesicle, containing the ingested material. The vesicles can then

either merge with other cellular membranes (to transport the materials inside the cell), with lysosomes (in which case the material is degraded), or with the basolateral membrane (in which case the material enters the blood stream).

A number of receptors are presented on the surface of cells (*e.g.* insulin receptor). These receptors can act as transporters (known as receptor-mediated transporters) or as modulators for transporter mechanisms.

1.2.4 Barriers to drug absorption

Many physiological factors affect the absorption of drugs. These include gastrointestinal motility, gastric emptying, the surface pH of the mucosa, the unstirred water layer, intestinal blood and lymph flow, and colonic microflora. The effects of pH and how it relates to the unstirred water layer are examined in more depth below.

The parietal cells of the stomach secrete hydrogen ions. When the acidic contents of the stomach reach the duodenum, it causes the secretion of a bicarbonate-rich alkaline fluid from the pancreas. This fluid, and other alkaline secretions, neutralises the acid entering the duodenum. In the small intestine the measured pH becomes progressively alkaline in the distal portions. These differences can be important in the absorption of weak acidic and basic drugs.

An acidic microlayer next to the intestinal mucosa has long been identified [Kakemi *et al.*, 1969]. This layer is approximately 20 μm thick and has a pH value that is 0.5 lower than the pH of the bulk phase (which has a pH of 6.2 [Fawcus *et al.*, 1997]). This acidity is potentially caused by the exchange of potassium and hydrogen ions across the mucosa. The acidic microclimate will affect the fraction of ionised drug present at the absorption site.

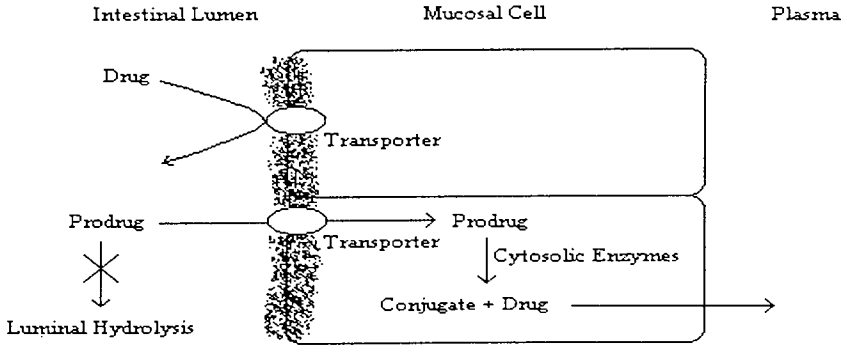
An unstirred water layer that is not in equilibrium with the bulk phase preserves the acidic microclimate and can be a rate-limiting step for drug absorption [Wilson & Dietschy, 1972]. This layer is approximately 300 μm thick. Drug molecules must penetrate this aqueous layer before reaching the hydrophobic phospholipid membrane. This aqueous layer can be a rate-limiting step for the absorption of lipophilic molecules.

When a drug has crossed the apical surface and entered a cell, it has to cross the basolateral membrane to leave the cell and enter the blood. In addition to physicochemical barriers, metabolic barriers to absorption can exist. Instead of leaving the cell the drug may be metabolised into a different compound or excreted back across the apical surface. Many cellular enzymes could metabolise the drug, including peptidases (breaks peptide bonds) and esterases (degrades esters). The cytochrome P450 family of enzymes can oxidise atoms in the drug to create a new functional group that can then be used to conjugate the drug metabolite to larger hydrophilic groups (such as sulphates). These enzymes are examined in more detail below (Section 1.4). Many efflux transporters have been identified. The most well known are p-glycoprotein, multidrug-resistance protein (MRP) and the organic anion transport protein (OATP). These transporters are examined in more detail below (Section 1.5).

1.2.5 The prodrug approach to overcome absorption barriers

The prodrug approach to improve drug absorption has generally been used to increase the lipophilicity of a drug and hence to improve its permeability. Prodrugs can be targeted at a specific enzyme to release the parent drug into the cell. A polar drug with a low permeability can be converted into a (polar) conjugated drug that is a substrate for the transporter. Following transport across the apical membrane, hydrolysis (by the enzyme) back to the parent drug would occur and the drug enter the blood stream [Amidon & Lee, 1994]. This approach is summarised in Figure 1.3 below. Examples of three peptide-based prodrugs (ACE inhibitors, Methyldopa, and Valacyclovir) are examined in Section 1.3.4 below).

Figure 1.3 Schematic representation of a prodrug strategy



Adapted from Fig. 5, Amidon & Lee, 1994

1.3 Peptide absorption

1.3.1 In vivo *peptide absorption*

For many years, it was thought that the absorption of protein digestion products amounted to little more than the absorption of a mixture of free amino-acids. Then it became clear that small peptides were also transported into the absorptive cells of the intestinal mucosa, although in general only free amino-acids enter the portal blood.

In the 1960s, a hypothesis about protein absorption was put forward. Proteins were partially hydrolysed in the intestinal lumen to peptides of various sizes, which then entered mucosal cells and were hydrolysed to amino-acids, which then entered the blood stream. In 1968, it was reported that amino-acids could be more rapidly absorbed from small peptides than from the equivalent mixture of free amino-acids. Not only was the total absorption of amino-acids greater from the peptides than from the equivalent free amino-acids, but also competition for absorption between two amino-acids was avoided when peptides of the two different amino-acids were absorbed [Matthews, 1975].

In vivo absorption of dipeptides has been used to treat many diseases including Hartnup disease and cystinuria. Hartnup disease is caused by a genetic defect in amino-acid transport. Free histidine and free tryptophan are not absorbed and the resulting deficiency causes skin lesions and impaired co-ordination. A study on the absorption of free and peptide-bound amino-acids in a patient with Hartnup disease found that many neutral amino-acids were poorly absorbed in the free form but more readily absorbed in when given as peptides [Leonard *et al.*, 1976]. One treatment proposed as a result of this study was the administration of two dipeptides, β-Ala-His and Gly-Trp, which were found to be absorbed normally and then hydrolysed to release the required amino-acids into the blood. Cystinuria is the presence of cystine (a cysteine S-S dimer) and dibasic amino-acids (*e.g.* lysine and arginine) in the urine. These amino-acids are poorly absorbed and the resulting deficiencies can be overcome by *in vivo* treatment with dipeptides (*e.g.* Gly-Lys or Arg-Asp) [Matthews, 1975].

1.3.2 Di/tri-peptide transport system

There is an obvious difficulty in obtaining satisfactory evidence for active transport of peptides due to their rapid hydrolysis. This means that most peptides cannot be detected

intact in the intestinal mucosa or in the blood. However, certain peptides with unusual structural features are relatively well transported but so poorly hydrolysed that they accumulate and can be detected. These unusual peptides include glycyl-sarcosine (Gly-Sar), carnosine, and β-alanyl-glycyl-glycine (β-Ala-Gly-Gly). Uptake of β-Ala-Gly-Gly was inhibited by anoxia, replacement of sodium ions, and the presence of some dipeptides. It was concluded that uptake was the result of a carrier-mediated energy-dependent process [Addisson *et al.*, 1975]. Further evidence for the dependence of peptide transport on metabolic energy was indicated by the inhibition of transport by hypoxia (lack of oxygen), ATP depletion (lack of metabolic energy), and metabolic inhibitors such as cyanide and dinitrophenol [Walter *et al.*, 1996a].

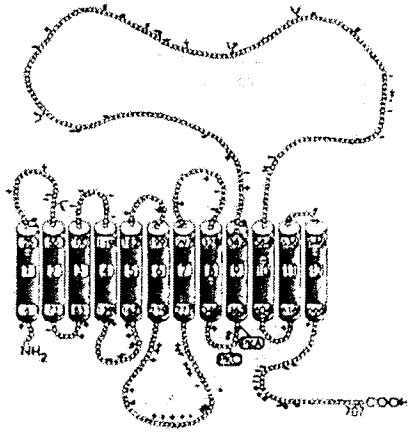
The molecules of many biologically active peptides were thought to be too large for extensive absorption by the peptide transporter. However, the existence of a limited degree of absorption of hormonal regulatory factors may be due to a transporter. One example is thyrotropin-releasing factor (pyroGlu-His-ProNH_2). This tripeptide derivative, which is very resistant to enzymic hydrolysis, is active in relatively large oral doses and is absorbed from the small intestine in man [Matthews, 1975].

Studies using an inward directed proton gradient showed that peptide transport was electrogenic (creates an electronic difference across the membrane), resulting in the transfer of a positive charge across the membrane. Gly-Sar transport in Caco-2 cells was greatest when the apical surface was acidified (*i.e.* there was a proton gradient across the membrane) [Thwaites *et al.* 1993]. A direct link between peptide transport and cytosolic acidification was also shown. The coupling of peptide transport with a proton gradient is strange as other nutrient transport systems use a sodium gradient. However, the two gradients separate the amino-acid and peptide transporters and allow them to operate independently [Walter *et al.*, 1996b].

Studies had shown that a carrier protein should exist so the next wave of studies tried to identify the specific protein. A rabbit intestinal cDNA library was cloned, using *Xenopus laevis* oocytes, and screened for an increased uptake of ^{14}C-labelled glycyl-sarcosine compared to control oocytes [Fei *et al.*, 1994]. A 707-amino-acid protein was isolated, identified as the rabbit proton-coupled transporter of proteins and called PepT1. Following the identification of the rabbit peptide transporter, a human intestinal cDNA library was screened using a probe derived from the rabbit transporter [Liang *et al.*, 1995]. A cDNA

fragment was identified and when it was expressed, in HeLa cells or in *Xenopus laevis* oocytes, there was an increase in proton-dependent peptide transport activity. The cDNA of the transporter was 2263 base pairs long with an open reading frame of 2127 base pairs. The predicted protein consisted of 708 amino-acids, a molecular size of 79 kDa, and twelve membrane-spanning domains. Chromosomal assignment studies located the gene to chromosome 13 q33-q34. The human H^+/peptide cotransporter gene was named hPEPT1 (see Figure 1.4 below for model).

Figure 1.4 Membrane model of PepT1



Putative membrane-spanning regions are depicted as cylinders. Adapted from Fei *et al.*, 1994

A different strategy to identify the relevant membrane protein was to use a monoclonal antibody that blocked the uptake of a β-lactam antibiotic (a substrate for the peptide transporter) into Caco-2 cells [Dantzig *et al.*, 1994]. A 92 kDa membrane protein was identified and designated HPT-1. It had one transmembrane domain and when expressed in Chinese Hamster Ovary (CHO) cells, the resulting transport activities were similar but not identical to endogenous peptide transport in Caco-2 cells. As transporter proteins commonly have multiple transmembrane domains, it was concluded that HPT-1 might associate with other proteins to become fully active [Adibi, 1997]. The relationship between hPEPT1 and HPT-1 is unclear.

In the intestine the peptide transporter PepT1 provides the major route for the absorption of the end-products of protein digestion. In renal proximal tubules, the homologous peptide transporter PepT2 plays an important rolein the reabsorption of filtered peptides [Barfuss *et al.*, 1988]. PepT2 is expressed in many tissues but not the intestine [Boll *et al.*, 1996].

Intracellular accumulation of peptides is reduced by the action of cytoplasmic peptidases. These enzymes hydrolyse the peptides into amino-acids. The amino-acids are then used by the cells or excreted out of the cells *via* amino-acid transporters located on the basolateral membrane. However, not all peptides are hydrolysed and so those peptides have to be dealt with. Recent studies have suggested the presence of a peptide transporter in the basolateral membrane. The transcellular flux of bestatin, a peptide-like anticancer agent, was inhibited in both directions (apical-to-basolateral and *vice versa*) by dipeptides [Saito & Inui, 1993]. Uptake from the apical surface had an optimum pH of 6.0 but uptake from the basolateral surface was not sensitive to pH. Kinetic parameters also indicated that the apical and basolateral transporters could be distinguished from each other. The physiological function of the basolateral transporter is probably to excrete dipeptides out of the cell. Intravenous injection of a dipeptide led to accumulation of the dipeptide in the kidney (where there is another transporter) but not the intestine. It was concluded that the basolateral peptide transporter does not absorb dipeptides from the blood [Adibi, 1997]. Two separate transporters imply a degree of differentiation in their regulation that is unclear at present.

A cDNA fragment of sheep PepT1 was used as a probe to study the distribution of peptide transporter mRNA in various tissues of sheep, cows, pigs and chickens [Chen *et al.*, 1999]. The mRNA was always found in the small intestine but not the liver or kidney of all the species. It was concluded that the peptide transporter is primarily an intestinal transporter.

1.3.3 In vitro *absorption of small peptides and peptidomimetics*

Peptides and peptidomimetic drugs can be defined as being composed of amino-acids or amino-acid analogues and whose synthesis is based on some analogy with natural proteins [Amidon & Lee, 1994]. The main classes of peptidomimetic drugs are the β-lactam antibiotics and the angiotensin-converting enzyme (ACE) inhibitors.

Intestinal absorption of captopril was examined in rats using the single-pass perfusion method [Hu & Amidon, 1988]. Dipeptides, dinitrophenol and cephradine inhibited absorption. There was also significant uptake *via* a passive mechanism. It was concluded that captopril is partially transported by the peptide transporter. This was the first study that linked ACE inhibitors to the peptide transporter.

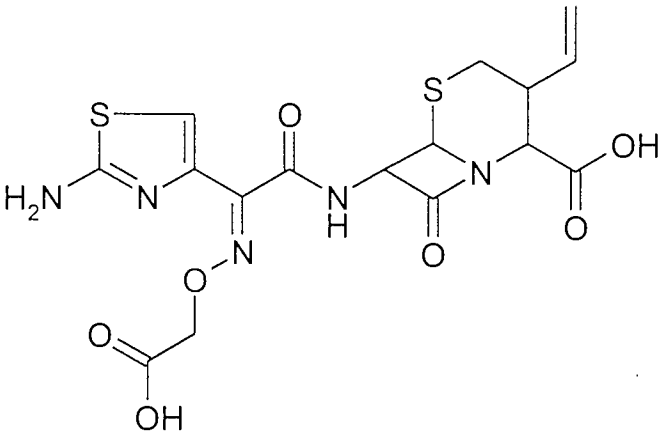
Amino β-lactam antibiotics include penicillin derivatives and cyclosporin derivatives. Some of the structures are shown in Figure 1.5 below. They have a higher absorption than the pH-partition theory would suggest [Smith *et al.*, 1992]. They resemble the structures of di- and tripeptides, *e.g.* D-Ala-D-Ala and Phe-Cys-Val. They have a free N-terminal α-amino group and a C-terminal carboxyl group. Their absorption is inhibited by di/tripeptides but not by amino-acids [Sinko & Amidon, 1989]. Uptake of cephalexin into Caco-2 cells was competitively inhibited by dipeptides and was also found to be stereospecific for the L-enantiomer [Dantzig & Bergin, 1990].

Intestinal absorption of cefdinir, cefixime and cephradine were investigated using rabbit brush-border membrane vesicles [Tsuji *et al.*, 1993]. Cefdinir uptake was inhibited by the presence of dipeptides (Gly-L-Pro and Gly-Sar), other β-lactam antibiotics (cephradine and penicillin V), and monocarboxylic acids (acetic and lactic). The uptake of cefixime and cephradine were both inhibited by the monocarboxylic acids. Cefdinir was also found to inhibit the uptake of cephradine, cefixime and acetic acid. It was concluded both the peptide and monocarboxylic acid transporters transported cefdinir. This shows that some drugs could use more than one transporter to enter the cell.

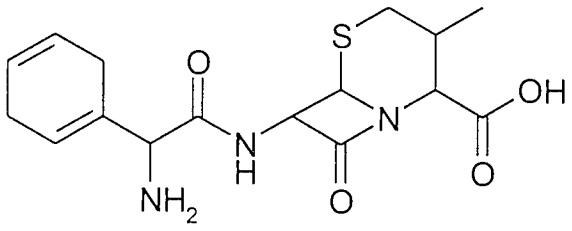
1.3.4 Peptide-based prodrugs: ACE inhibitors, Methyldopa & Valacyclovir

The broad specificity exhibited by the peptide transporter, and the presence in the cytoplasm of prolidase (an enzyme which breaks peptide bonds containing proline), suggested that prodrugs could be targeted at the transporter. Prolidase is a cytosolic enzyme found in many cells that hydrolyse iminodipeptides (X-Pro) as well as the amino-acid proline [Bai *et al.*, 1992]. It has been shown that the enzyme will hydrolyse dipeptides as long as they have an N-terminal α-amino group. Prodrugs containing a peptide bond can enter the cell using the peptide transporter where they are then metabolised by prolidase or other cytosolic enzymes to release the active metabolites.

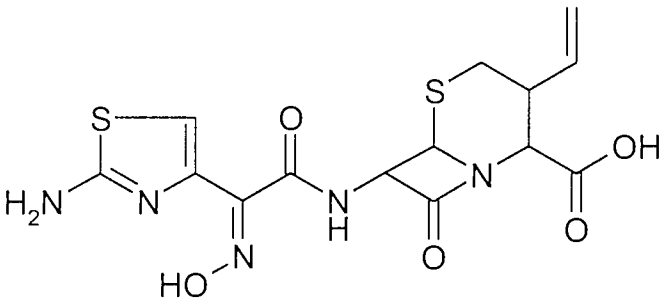
Figure 1.5 The structures of some β-lactams and similar peptides (neutral forms)



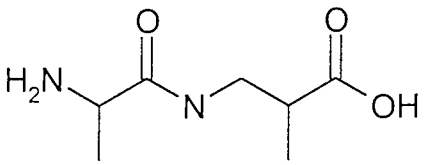
Cefixime



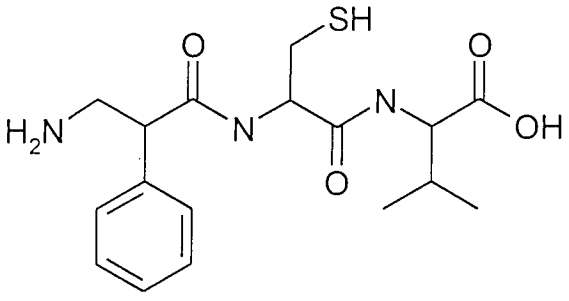
Cephradine



Cefdinir



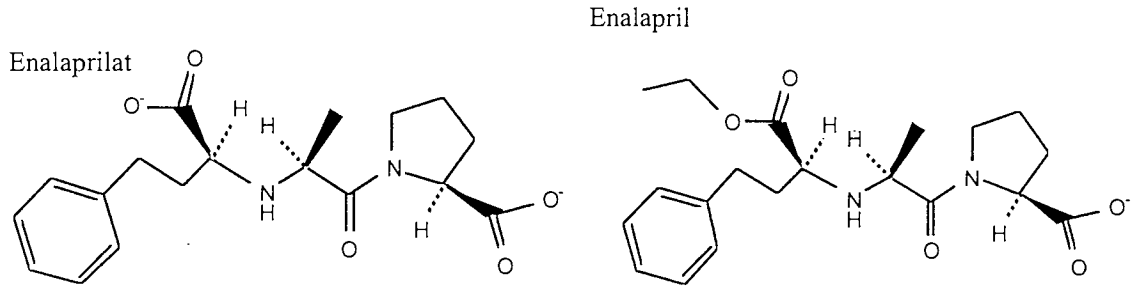
Ala-Ala



Phe-Cys-Val

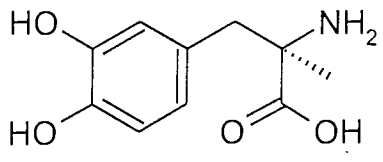
Angiotensin-converting enzyme (ACE) cleaves the inactive protein angiotensin I into the active vasoconstricting protein angiotensin II. Drugs that inhibit this enzyme are known as ACE inhibitors and are used to treat hypertension and congestive heart failure. Known ACE inhibitors include enalapril, captopril and fosinopril. These are all prodrugs, esters of the active compounds. Enalaprilat is the active dipeptide but has a low permeability and a low systemic availability. Enalapril is the ethyl ester of enalaprilat, is well absorbed, and is hydrolysed back to the active drug *in vivo* [Amidon & Lee, 1994]. The two structures are shown in Figure 1.6 below.

Figure 1.6 The structures of Enalaprilat and Enalapril



A poorly absorbed amino-acid analogue is α-methyldopa (see Figure 1.7 below). This is an anti-hypertensive agent used to lower blood pressure. Dipeptides containing this compound had a significantly increased permeability compared to pure α-methyldopa [Hu *et al.*, 1989]. The peptides were hydrolysed in the cells, resulting in an increased systemic availability of α-methyldopa. This was an early study showing the broad substrate specificity of the peptide transporter as well as indicating the success of a prodrug strategy to increase drug absorption.

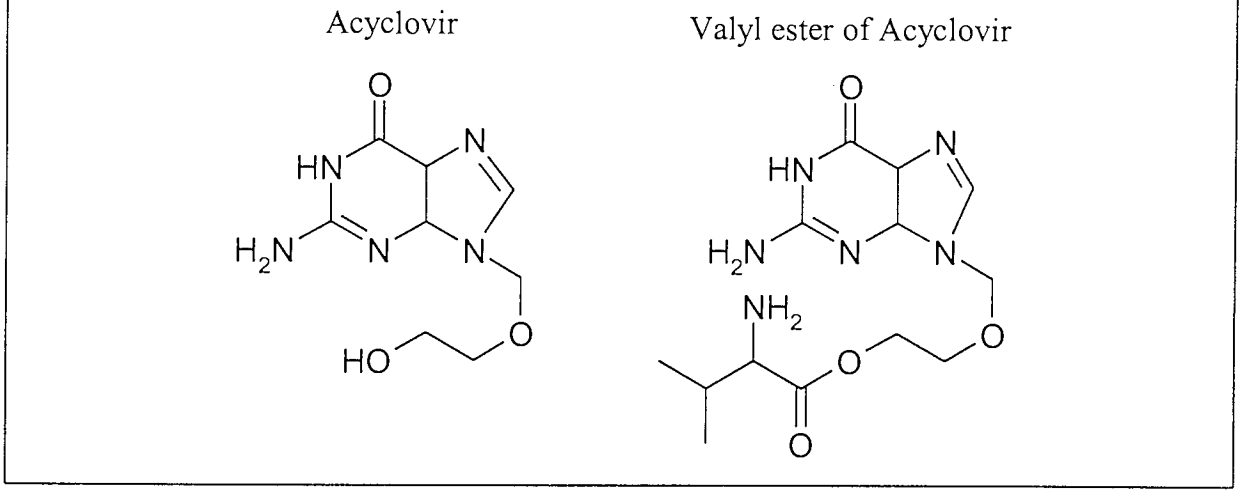
Figure 1.7 The structure of L-α-Methyldopa



More than 90% of patients with HIV are infected at some time by cytomegalovirus (CMV) and up to 40% of those with advanced HIV diseases develop CMV infections. Also, patients with AIDS are at a high risk of developing progressive or recurrent mucocutaneous herpes simplex virus, varicella zoster virus and other AIDS opportunistic

diseases [Baliman *et al.*, 1998]. It is therefore essential to have sufficient plasma levels of antiviral agents after oral administration, in order for any treatment to be effective. The acyclic nucleoside acyclovir (ACV) was the first anti-herpetic agent to selectively inhibit herpes virus replication, while maintaining an excellent safety profile. However, its low oral bioavailability led to the development of the prodrug valacyclovir (Val-ACV), which is the L-valyl ester of ACV. The structures of ACV and Val-ACV are shown in Figure 1.8. Due to the high plasma levels of ACV after the administration of Val-ACV, *in vivo* activity against herpes simplex virus types 1 and 2 and varicella zoster virus, can be achieved [Baliman *et al.*, 1998].

Figure 1.8 The structure of acyclovir and its valyl ester



The transepithelial transport of Val-ACV was investigated using Caco-2 cells [de Vrueh *et al.*, 1998]. Apical-to-basolateral transport was seven-fold faster than for ACV, and L-Val-ACV was twice as permeable as D-Val-ACV [Han *et al.*, 1998]. Gly-Sar but not L-Val inhibited transport. Val-ACV also inhibited the uptake of cephalexin. Val-ACV uptake was concentration-dependent and was inhibited by Gly-Sar and cefadroxil. CHO cells transfected with hPEPT1 took up Val-ACV at an optimum pH of 7.5 [Guo *et al.*, 1999]. These results suggested that Val-ACV was a substrate for the peptide carrier PEPT1. After transport, the receiver solution contained 90% ACV. It was concluded that Val-ACV is a prodrug for ACV that releases the drug after absorption into the cells.

1.3.5 Summary of peptide absorption

For many years it was thought necessary for proteins to be hydrolysed to amino-acids before they could be absorbed in the intestine, however later research found that dipeptides

could be absorbed into cells where they were digested and excreted into the blood as amino-acids. This led to the discovery of the dipeptide transporter (which also transported tripeptides) and its gene, hPEPt1. A number of peptidomimetic drugs have been found to be substrates for the transporter, including the ACE inhibitors and β-lactam antibiotics. This broad range of substrates led to the development of prodrugs that targeted the transporter; however there has been little research on the optimum structural features required by prodrugs to be transporter substrates.

1.4 Metabolism as a barrier to absorption

Metabolism is a process by which the molecular structure of a compound is altered by an enzyme. Metabolism as a barrier to drug absorption is where the molecular structure of easily absorbed lipophilic compounds are converted to a more hydrophilic nature to reduce their absorption. There are two stages of metabolism. Phase I is the creation of a functional group in the molecule and Phase II is the conjugation of a hydrophilic group to the functional group in the molecule. The major Phase I reactions are oxidation, reduction and hydrolysis, the majority of which are catalysed by the cytochrome P450 mono-oxygenase system containing cytochrome P450s and a number of other enzymes.

1.4.1 Cytochrome P450

Cytochrome P450s belong to a group of similar proteins (or isozymes) that have the same porphyrin-haem complex (iron-containing pigment) at the catalytic centre but different amino-acid sequences resulting in different conformations of the active site. As the P450s exist as a family of enzymes, their amino-acid sequences form the basis of their classification and naming. In general, if P450s exhibit more than 40% amino-acid sequence homology, they belong to the same family whilst those displaying more than 60% amino-acid sequence homology belong to the same subfamily [Spatzenegger and Jaeger, 1995]. Each isoform has a name derived from the fact it is a cytochrome P450 (CYP), its family (number) and its subfamily (letter). For example, CYP2D6 was the sixth gene product to be identified as a member of the D subfamily of the 2 family of Cytochrome P450.

The distribution of cytochrome P450s along the gastrointestinal tract is not uniform. In the oesophagus, stomach and colon, there are minimal amounts of CYP3A, CYP2E1, CYP2D6, CYP1A2 and CYP2C whilst the small intestine contains much higher amounts

of CYP3A, CYP2D6 and CYP2C. Within the small intestine, the duodenum and jejunum contain a higher total amount of mucosal P450 compared to the ileum. Transition from the ileal to colonic mucosa results in a further drop of total amount of P450 content [de Wazier *et al.*, 1990]. The microsomal enzymes in the small intestine contain approximately 50% CYP3A4 and 10-20% CYP2D6 of their respective levels in human liver [de Wazier *et al.*, 1990]. Therefore, therapeutic drugs that are absorbed at different sites in the small intestine are metabolised to different extents; this is one of many factors thought to contribute to their variable oral bioavailability.

1.4.2 CYP3A

Of several human P450 enzymes, those of the CYP3A sub-family have major importance since they are by far the most abundant of all the human CYP isoforms, have a broad substrate specificity, are localised in organs of particular relevance to drug disposition such as the gastrointestinal tract, kidney and the liver and their catalytic activity is readily modulated by a variety of compounds. There are at least three existing functional CYP3A proteins in humans, namely CYP3A4, CYP3A5 and CYP3A7.

CYP3A enzymes account for approximately 70% of total P450 enzymes in the jejunal mucosa [Watkins *et al.*, 1987]. Their distribution along the small human intestine is such that the level of microsomal CYP3A content is greatest in the duodenum and lowest in the ileum (931 *vs.* 17 pmol/mg of protein) [Paine *et al.*, 1997]. Similarly, CYP3A catalytic activity as reflected the metabolism of midazolam decreases from the duodenum to the jejunum to the ileum (644 *vs.* 426 *vs.* 68 pmol/min/mg of protein) [Paine *et al.*, 1997].

1.4.2.1 Mechanism of action

The haem iron in cytochrome P450 is usually in the ferric state (Fe^{3+}) and when reduced to its ferrous state (Fe^{2+}), the enzymes can bind to ligands such as oxygen (O_2) or carbon monoxide (CO). The complex formed absorbs light maximally at 450nm from which P450s derive their names [Parkinson, 1996]. Since P450s are mono-oxygenases, they oxidise compounds on carbon atoms or on heteroatoms such as nitrogen and sulphur [Parkinson, 1996]. A typical reaction of P450s is shown in Equation 1.3 below:

Equation 1.3 Example P450 reaction

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

Where:

RH – Reduced reactant compound containing a hydrogen atom

NADPH – Electron/Hydrogen donator (nicotinamide adenine diphosphate)

ROH – Oxidised reactant compound containing a hydroxyl group

$NADP^+$ - Reduced form of NADPH

The reaction requires nicotinamide adenine diphosphate (NADPH). This is used to relay electrons to P450s *via* another protein, and oxygen. The nature of the protein, that uses NADPH, varies depending on the subcellular location of P450s [Parkinson, 1996]. In the endoplasmic reticulum, where most P450s involved in the metabolism of xenobiotics are located, a flavoprotein called NADPH-cytochrome P450 reductase is involved. This enzyme must be imbedded in the phospholipid bilayer of the endoplasmic reticulum in order for it to function.

1.4.2.2 CYP3A3, CYP3A4 and CYP3A5

In the human intestine, there are two different but closely related intestinal CYP3A isoforms, namely CYP3A4 and CYP3A5 [Lown *et al.*, 1994]. CYP3A5 was found in 70% of intestinal biopsies [Lown *et al.*, 1994] but CYP3A4 was the most abundant CYP3A enzyme by far in the gastrointestinal tract [de Wazier *et al.*, 1990]. CYP3A3 is a very closely related isoform to CYP3A4 (>98% cDNA sequence similarity) but it is not known whether this reflects a separate genetic product or an allelic variant [Thummel and Wilkinson, 1998]. Therefore, the term CYP3A4 is generally taken to indicate a collective contribution of the two isoforms. CYP3A4 is located in mature enterocytes [Kolars *et al.*, 1992] but not in crypt cells. Its cellular distribution is such that it is prominent at the villus tip and forms an intense band at the apex of the cells as revealed by immunohistochemistry [Kolars *et al.*, 1992]. In parallel with protein expression, its gene is also detected in human enterocytes and is inducible by rifampicin [Kolars *et al.*, 1992, Lown *et al.*, 1996]. The interpatient heterogeneity in the expression of CYP3A4 and CYP3A5 in the small bowel has been well documented [Kolars *et al.*, 1991; Lown *et al.*, 1994, Watkins, 1994]. There

was a 6-fold variation in CYP3A catalytic activity as illustrated by midazolam hydroxylation, an 11-fold variation in the CYP3A4 protein content and an 8-fold variation in CYP3A4 mRNA content in human intestinal biopsies [Lown *et al.*, 1994]. Expression of CYP3A4 as reflected by the formation of 1-hydroxy/4-hydroxymidazolam, CYP3A4 protein and CYP3A4 mRNA varies with gender, with females having a higher expression than males [Lown *et al.*, 1994].

Cytochrome P450 3A5 contains 502 amino-acid residues with a calculated mass of 57,115 Da and displays 84% similarity with the amino-acid sequence of CYP3A4, the molecular weight of which is 52,000 Da [Aoyama *et al.*, 1989; Wrighton *et al.*, 1989]. Due to very similar molecular weights and amino-acid sequences, it is very difficult to distinguish CYP3A4 from CYP3A5 by Western blotting. Generally the term CYP3A4 is taken to include CYP3A5 as well.

1.4.2.3 Tissue distribution / location

CYP3A4 is universally found in the liver where it constitutes a major isoform, on average about 30% of the total CYP protein [Shimada *et al.*, 1994]. Apart from the liver, relatively high levels of CYP3A (about 50% of hepatic levels and 70% of total CYP protein) are also present in the gastrointestinal tract, particularly on the surface of mature enterocytes [Kolars *et al.*, 1992]. In the kidney, CYP3A4 is present in only 30% of renal tissue samples, mainly in the collecting ducts [Schuetz *et al.*, 1992; Haehner *et al.*, 1996], the reasons for such polymorphic expression are not currently understood.

CYP3A5 is expressed in about 10-30% of hepatic samples and at levels of 10-30% of CYP3A4 [Wrighton *et al.*, 1989; Wrighton *et al.*, 1990]. CYP3A5 is the predominant 3A isoform that is universally expressed in the kidney [Schuetz *et al.*, 1992]. It is also expressed heterogeneously in the gastrointestinal tract but in lower amounts than CYP3A4 except in the parietal cells of the stomach [McKinnon *et al.*, 1992] and in the colon [Gervot *et al.*, 1996].

The fourth CYP3A isoform is CYP3A7, which was originally found in foetal liver but may also be selectively expressed in adult liver at lower amounts than CYP3A4 and CYP3AS [Schuetz *et al.*, 1994].

1.4.2.4 Substrates, inducers and inhibitors

There are a number of compounds which have be discovered to be substrates for CYP3A. Table 1.1 below lists compounds that are substrates, inducers and inhibitors of CYP3A4 and CYP3A5. These include a number of families of compounds such as calcium-channel blockers and HIV protease inhibitors. The effect of these families of drugs on other proteins will be investigated later in this thesis.

1.4.3 Summary of metabolism as a barrier to absorption

Metabolism by cellular enzymes is a significant barrier to drug absorption for some drugs. The cytochrome P450 family of enzymes play a significant role in the intestine and liver. However, it is mainly the CYP3A4 isoform that plays a major role, as it is located on the surface of mature enterocytes. There are a huge number of modulators of CYP3A4 activity that include a number of families of drugs that have been intensively investigated.

Table 1.1 Substrates of CYP3A

Benzodiazepine Anxiolytic

Alprazolam
Clonazepam
Diazepam
Midazolam
Triazolam

Calcium Channel Blockers

Diltiazem
Nicardipine
Nifedipine
Pranidipine
Verapamil

HIV Protease Inhibitors

Indinavir
Ritonavir
Saquinavir

Other Compounds

17-α-Ethinyl oestradiol
17-β-Estradiol
Alfetanil
Amiodarone
Benzphetamine
Busipirone
Caffeine
Cannabidiol
Carbamazepine
Cisapride
Cocaine
Codeine
Colchicine
Corticosterone
Cortisol
cyclosporin A
Dantrolene

Other Compounds (continued)

Dapsone
Dextramethasone
Digitoxin
Enalapril
Erythromycin
Estrogens
Ethosuximide
Ethylmorphine
Etoposide
Flutamide
Glyceryl trinitrate
Ifosamide
Ketoconazole
Lidocaine
Mephenytoin
Miconazole
Oxodipine
Paclitaxel (Taxol)
Paracetamol
Praziquantel
Prednisone
Propafenone
Quinidine
Rapamycin
Retinoic Acid
Tacrolimus
Tamoxifen
Teniposide
Terfenadine
Testosterone
Theophylline
Toltzuril
Vinblastine
Warfarin
Zolpidem
Zonisamide

Adapted from Spatzenegger & Jaeger, 1995

1.5 Intestinal efflux mechanisms as a barrier to absorption

1.5.1 P-glycoprotein (PGP)

The development of multidrug resistance (MDR) is a major obstacle in the chemotherapeutic treatment of many human cancers. Resistant tumours are found to be cross-resistant to a broad but well defined spectrum of unrelated drugs including the *Vinca* alkaloids, anthracyclins and taxanes. Cells may become resistant by many mechanisms but one major type of MDR is linked to the overexpression of p-glycoprotein.

P-glycoprotein is a 170 kDa mammalian plasma membrane protein belonging to the ATP-binding cassette (ABC) superfamily. It is a tandemly duplicated molecule, with the two highly similar halves each possessing six transmembrane helices and an ATP-binding site [Gotesman & Pastan, 1993]. The genes for p-glycoprotein from hamsters, mice and humans have been cloned and sequenced, and homologues have been identified in many other species. It is encoded by a small multigene family (*mdr* class I, II and III). Humans express the class I isoform, which confers multidrug resistance, and the class III isoform, which is a phosphatidylcholine translocase to transport phosphatidylcholine into bile. It is the product of the *mdr*1 gene that will be focused on.

Experiments with purified p-glycoprotein reconstituted into proteoliposomes [Shapiro & Ling, 1995] have conclusively shown that p-glycoprotein is an ATP-dependent multidrug transporter with a remarkedly broad specificity for substrates.

There are two ATP-binding sites in the molecule, one in each half. They bind and hydrolyse ATP with a similar efficiency and can operate independently although they appear to switch to an alternating mode of operation when they interact with each other [Sharom, 1997].

1.5.1.1 Mechanism of action

The classic model of the action of membrane transporters is that the binding site is exposed to the aqueous phase (*e.g.* cytosol) and that the substrate is released into the aqueous phase on the other side of the membrane (*e.g.* extracellular medium). A conformational change induced by the binding of the substrate or ATP leads to the formation of a hydrophilic path

through the membrane so the substrate does not come into contact with membrane lipids. Many ABC transporters act this way however p-glycoprotein does not.

As many substrates for p-glycoprotein are hydrophobic (see Section 1.5.1.4), access to the substrate-binding site may be from the lipid bilayer rather than the aqueous phase. It has been suggested [Higgins & Gottesman, 1992] that p-glycoprotein acts as a "hydrophobic vacuum cleaner", removing drugs from the plasma membrane rather than the aqueous phase.

Structural modelling of p-glycoprotein [Pawagi *et al.*, 1994] has suggested there was one large, complex substrate-binding site composed of aromatic amino-acids found in several transmembrane helices. The reaction of photoaffinity substrate analogues to p-glycoprotein has also led to the suggestion that transmembrane helices from both halves form a single binding domain [Gotesman & Pastan, 1993]. However, the kinetics of rhodamine-123 and Hoechst 33342 in isolated p-glycoprotein-rich plasma membrane vesicles [Shapiro & Ling, 1997] challenged this view. Each substrate stimulated the transport of the other. Colchicine and quercetin stimulated the transport of rhodamine-123 but inhibited Hoechst 33342 transport. Daunorubicin and doxorubicin stimulated Hoechst 33342 transport but inhibited the transport of rhodamine-123. Vinblastine and etoposide inhibited the transport of both dyes. The results led to the conclusion that there were at least two positively cooperating sites for drug binding and transport.

P-glycoprotein is unusual among other ATPases in that the purified protein exhibits a high level of constitutive ATPase activity in the absence of substrates [Shapiro & Ling, 1995]. One explanation is that in p-glycoprotein the ATPase activity may be partially uncoupled from substrate binding and transport. The ATPase activity can be affected by a number of drugs, being stimulated at low concentrations and inhibited at higher concentrations, but conflicting results are obtained using the same drug in different cells lines [Sharom, 1997].

Fluorescence experiments using purified p-glycoprotein have established the existence of a conformational change induced by the binding of several drugs. The binding of a drug leads to a change in the conformation of the ATP-binding site, as seen by the quenching of a fluorescent probe bound there [Liu & Sharom, 1996].

The unusual nature of the p-glycoprotein transporter has led to the suggestion that it acts as a translocase or flippase, moving substrates from the inner leaflet to the outer leaflet of the membrane [Higgins & Gottesman, 1992]. Since the drug in each leaflet is in equilibrium with the aqueous phase, the presence of different amounts of drug within each leaflet would generate a drug concentration gradient across the membrane. The net effect of drug translocation within the membrane would be the same as expected for a classical membrane pump, as would the net effect of p-glycoprotein moving the drug form the inner leaflet to the extracellular aqueous phase directly. Since hydrophobic substrates have an innate ability to flip from one leaflet to the other, while p-glycoprotein is flipping the substrate in one direction some of the substrate will be flipping back. The net movement of the substrate (what is measured experimentally) will not represent the actual rate of turnover of the transporter. One study [Shairo & Ling, 1995] found that the apparent rate of Hoechst 33342 transport was 50-fold slower than the rate of ATP hydrolysis. This discrepancy could be due to a high innate ATPase activity and the difference between p-glycoprotein activity and the net efflux of the substrate.

1.5.1.2 Tissue distribution / location

P-glycoprotein is also found in several normal human tissues, including the apical surface of epithelial cells and the endothelial cells of the blood-brain-barrier. It is expressed at a high level in the kidney, a moderate level in the lung, liver and colon, and weakly or not expressed in most other tissues [Fojo *et al.*, 1987]

1.5.1.3 Substrates

P-glycoprotein is an unusual ABC protein in that it appears to be highly promiscuous. Some substrates, inducers and inhibitors of p-glycoprotein are shown in Table 1.2 below. A "typical" substrate is large (M_r >400), hydrophobic, amphipathic, with a planar ring system, and often has a positive charge at physiological pH [Pearce *et al.*, 1989]. However, many substrates are uncharged at physiological pH and several uncharged cyclic and linear hydrophobic peptides are also substrates.

Many of the inhibitors of p-glycoprotein are also substrates and therefore competitively inhibit the transporter. Some inhibitors are transported by p-glycoprotein (*e.g.* verapamil) but MDR cells are not resistant to those compounds.

Table 1.2 Substrates, inducers and inhibitors of p-glycoprotein

Name	Compound	PGP			Source
		Substrate	Inducer	Inhibitor	
Amlodipine	Calcium Channel Blocker			YES	A
Bepridil	Calcium Channel Blocker			YES	B
Diltiazem	Calcium Channel Blocker	YES	YES	YES	B, C
Felodipine	Calcium Channel Blocker			YES	B
Loperamide	Calcium Channel Blocker	YES			B
Nicardipine	Calcium Channel Blocker	YES	YES	YES	A, B, D
Nifedipine	Calcium Channel Blocker		YES	YES	B, C, D
Nimodipine	Calcium Channel Blocker			YES	D
Nitrendipine	Calcium Channel Blocker			YES	B, D
Verapamil	Calcium Channel Blocker	YES	YES	YES	B, C, D, E
Actinomycin D	Antibiotic	YES			B, F
Erythromycin	Antibiotic	YES	YES	YES	B, C
Gramicidin D	Antibiotic	YES			F
Mithramycin	Antibiotic	YES			F
Mitomycin C	Antibiotic	YES			F
Oligomycin	Antibiotic			YES	E
Puromycin	Antibiotic	YES			F
Rapamycin	Antibiotic	YES	YES	YES	B, C
Rifampin	Antibiotic		YES		G
Amprenavir	HIV Protease Inhibitor	YES			B
Indinavir	HIV Protease Inhibitor	YES			B, F
Nelfinavir	HIV Protease Inhibitor	YES			B
Ritonavir	HIV Protease Inhibitor	YES		YES	B, F
Saquinavir	HIV Protease Inhibitor	YES			B, F
Chlorambucil	Anti-cancer drug	YES	YES	NO	B, E
Fluorouracil	Anti-cancer drug	YES	YES		B
Hydroxyurea	Anti-cancer drug	YES	YES		B
Paclitaxel /Taxol	Anti-cancer drug	YES			B, C, F
Atorvastatin	HMG-CoA inhibitor	YES		YES	B, H
Lovastatin	HMG-CoA inhibitor			YES	C, H
Pravastatin	HMG-CoA inhibitor			NO	H
Simvastatin	HMG-CoA inhibitor			YES	C, H
Daunorubicin	Anthracycline	YES	YES		B, F
Doxorubicin	Anthracycline	YES	YES		B, F
Epirubucin	Anthracycline	YES			F

Table 1.2 Substrates, inducers and inhibitors of p-glycoprotein (continued)

Name	Compound	PGP			Source
		Substrate	Inducer	Inhibitor	
Cortisol	Glucocorticoid	YES		YES	B, C, I
Corticosterone	Glucocorticoid	YES		YES	C, I
Dexamethasone	Glucocorticoid	YES	YES	YES	B
Cimetidine	Histamine receptor antagonist	YES			B
Ranitidine	Histamine receptor antagonist	YES			B
Terfenadine	Histamine receptor antagonist			YES	B, C
Clotrimazole	Potassium channel blocker		YES		B
Quinidine	Potassium channel blocker	YES		YES	B, C
Quinine	Potassium channel blocker			YES	B
6',7'-dihydroxy bergamottin	Furanocoumarin			YES	J
Bergamottin	Furanocoumarin			Y/N	J
Bergapten	Furanocoumarin			YES	K
Phenobarbitone	Anti-convulsant		YES		B
Phenytoin	Anti-convulsant	YES	YES		B
Itraconazole	Anti-fungal	YES		YES	B
Ketoconazole	Anti-fungal	YES		YES	C
Etoposide	Epipodophyllotoxin	YES	YES		B, C, F
Teniposide	Epipodophyllotoxin	YES			C, F
Calcein-AM	Dye Derivative	YES			F
Rhodamine-123	Dye	YES			F
Naringenin	Flavone derivative			NO	J, K
Naringin	Flavone derivative			YES	J, K
Methadone	Narcotic analgesic	YES			B
Morphine	Narcotic analgesic	YES	YES		B
Amitripyline	Tricyclic antidepressant	YES			B
Nortryptaline	Tricyclic antidepressant	YES		YES	B
Vinblastine	Vinca alkaloid	YES	YES	YES	B,C, D, E, F
Vincristine	Vinca alkaloid	YES	YES		B, F
Aldosterone	Mineralocorticoid	YES		YES	I
Amiodarone	Ion channel blocker		YES	YES	B
Astemizole	-			YES	B
Benzbromarone	-			NO	E
Bromocriptine	Dopamine receptor agonist		YES		B
Celeprolol	-	YES			B
Chlorpromazine	Anti-psychotic			YES	B

Table 1.2 Substrates, inducers and inhibitors of p-glycoprotein (continued)

Name	Compound	PGP			Source
		Substrate	Inducer	Inhibitor	
Cisapride	-	NO		YES	L
Cisplatin	Immunotherapeutic	YES	YES		B
Clarithromycin	-			YES	B
Colchicine	Anti-mitotic	YES	YES		B
cyclosporin A	-	YES	YES	YES	B,C,E,F
Cytarabine	DNA synthesis inhibitor	YES			B
Digoxin	-	YES			B
Dipyridamole	Adenosine transport inhibitor			YES	B
Disulfiram	-			YES	B
Docetaxel	-	YES			B
Domperidone	Dopamine receptor antagonist	YES			B
Econazole	-			YES	E
Emitine	Apoptosis inducer	YES			F
Ethidium Bromide	DNA binder	YES			F
Fentanyl	Analgesic	YES			B
Fexofenadine	-	YES			B
Genistein	Protein kinase inhibitor			NO	E
Indomethacin	Cyclooxygenase inhibitor			NO	E
Insulin	Peptide hormone		YES		B
Lidocaine	Sodium channel blocker			YES	C
Losartan	-	YES			B
Methotrexate	Folic acid antagonist	YES	YES		B
Methylprednisolone	-	YES			B
Midazolam	Benzodiazepine anxiolytic		YES	YES	B, C
Mitoxantrone	DNA intercalator	YES	YES		B
Octreotide	-	YES			B
Ondansetron	-	YES			B
Phenothiazine	-		YES		B
Probenecid	-		YES	NO	B, E
Progesterone	Steroid	NO		YES	B
Quercetin	-			NO	J
Reserpine	-		YES	YES	B
Sodium Cholate	Detergent			NO	E
Sparfloxacin	-	YES			B
St. John's Wort	Herbal plant		YES		B
Sulfinpyrazone	-			NO	E

Table 1.2 Substrates, inducers and inhibitors of p-glycoprotein (continued)

Name	Compound	PGP			Source
		Substrate	Inducer	Inhibitor	
Tacrolimus	-	Y/N	YES	YES	B
Talinolol	-	YES			B
Tamoxifen	Protein kinase C inhibitor	YES	YES	YES	B, E
Testosterone	Androgen	YES		YES	C
Tetrabenazine	-			YES	B
Topotecan	-	YES			B, F
Valinomycin	Potassium ionophore	YES		YES	B, F
Yohimbine	Adrenergic receptor antagonist		YES		B

Source references:

A - Katoh *et al.*, 2000
B – Matheny *et al.*, 2001
C – Spatzenegger & Jaeger, 1995
D - Pascaud *et al.*, 1998
E - Hollo *et al.*, 1996
F - Ambudkar *et al.*, 1999
G - Weber *et al.*, 2001
H - Wang *et al.*, 2001a
I – Ueda *et al.*, 1997
J - Eagling *et al.*, 1999
K - Ho *et al.*, 2000
L – Abdel-Rahman *et al.*, 2000

It has been proposed that all the substrates and inhibitors are transported after the hydrolysis of ATP [Eytan *et al.*, 1996]. Compounds will naturally "flip" into the outer leaflet of the plasma membrane and "flop" back into the inner leaflet. The net transport of a compound will be depend on the rate at which drugs flip-flop across the membrane. The known substrates of p-glycoprotein were found to equilibrate across lipid bilayers relatively slowly (minutes to hours) whereas known inhibitors crossed the bilayers too rapidly to measure experimentally (seconds). For substrates, the transmembrane movement is presumably slow enough that p-glycoprotein can keep pace and create a net movement of the compound. For inhibitors, the transmembrane movement is so rapid that p-glycoprotein cannot keep pace with it. The result is a high turnover of p-glycoprotein but no concentration gradient will form and the cells will not be resistant to those compounds.

Alternative mechanisms for inhibitors of p-glycoprotein include ATPase inhibitors and membrane fluidisers (which would increase the rate of compounds "flopping" back to the inner leaflet). Neither group of inhibitors would need to be able to bind to p-glycoprotein.

As you can see from Table 1.2, there are a huge variety of compounds that are substrates and/or inducers and/or inhibitors. Although the magnitude of effects and affinity for the transporter are not shown in Table 1.2, the source references quoted do show the variety among the compounds. It can also be seen that small changes to chemical structures can abolish activity *e.g.* pravastatin is not a substrate while all the related HMG-CoA inhibitors (including simvastatin) are. The structural difference between pravastatin and simvastatin are a methyl group instead of a hydroxyl group and different molecular arrangements of a seven-carbon side-chain. There is a huge potential for drug-drug interactions as many structurally unrelated compounds are substrates for p-glycoprotein. There are also a large number of experiments to be performed to fill in the gaps in the current knowledge.

1.5.1.4 Summary of p-glycoprotein

The development of multidrug resistance in cancer chemotherapy resulted in the identification of p-glycoprotein. This efflux transporter is a plasma membrane ATP-dependent transporter with a very broad specificity for (mainly hydrophobic) substrates. There are a huge range of substrates and inhibitors of p-glycoprotein implying a huge potential for drug-drug interactions but also the huge potential for modulation of drug absorption.

1.5.2 Multidrug Resistance Protein (MRP)

The excretion of xenobiotics from intestinal epithelial cells cannot be accounted for by p-glycoprotein alone. A number of studies showed that there is intestinal excretion of the anionic glucuronide conjugates of a number of compounds, for example 1-naphthol [de Vries *et al.*, 1989]. P-glycoprotein transports cationic or neutral compounds [Pearce *et al.*, 1989] therefore other transporters must be responsible.

The doxorubicin-selected human small-cell lung cancer cell line H69AR was found to be resistant to many agents but did not over-express p-glycoprotein [Cole *et al.*, 1992]. The cell line did over-express mRNA for a different ATP-binding cassette transporter, later called MRP1. Other related proteins were then identified (MRP2 – MRP6) including MRP2 also known as the canalicular multispecific organic anion transporter (cMOAT) [Buchler *et al.*, 1996].

Human MRP1 is a large molecule (~200 kDa) and contains 1531 amino-acids, and although the amino-acid sequence of MRP1 indicated that it was a member of the ATP-binding cassette transporter family, its primary structure was not closely related to that of p-glycoprotein [Cole *et al.*, 1992]. Like p-glycoprotein, it had membrane-spanning domains and nucleotide-binding domains. However, it also had an extremely hydrophobic N-terminal region containing five or six potential transmembrane sequences [Stride *et al.*, 1996]. This feature is shared with other ABC transporters such as the sulfonylurea receptor but not the family as a whole. The genes for MRP1 and MRP6 have been localized to chromosome 16 although the genes for the other members of the family are scattered throughout the genome (MRP2 on chromosome 10, MRP3 on chromosome 17, MRP4 on chromosome 13 and MRP5 on chromosome 3) [Lecureur *et al.*, 2000].

1.5.2.1 Mechanism of action

MRP1 was found to be responsible for the excretion of the endogenous glutathione S-conjugate leukotriene C_4 from inside-out membrane vesicles [Leier *et al.*, 1994]. This led to the conclusion that the function of MRP1 was to be the ATP-dependent membrane transporter for glutathione and other anionic conjugates.

Drug uptake experiments using inside-out membrane vesicles prepared from NIH 3T3 cells expressing MRP1 were performed to investigate the biochemical mechanism by which MRP1 confers drug resistance [Paul *et al.*, 1996]. ATP-dependent transport was observed for a number of lipophilic cytotoxic agents including daunorubicin, etoposide and vincristine although only a minor effect was observed with taxol and vinblastine. Daunorubicin transport was competitively inhibited by reduced and oxidised glutathione as well as glutathione conjugates. It was concluded that MRP1 pumps unchanged lipophilic cytotoxic drugs out of cells leading to multidrug resistance. Glutathione was thought to be a co-transported substance and therefore necessary for transport of unconjugated drugs.

1.5.2.2 Tissue distribution / location

Northern blotting for MRP1 detected its mRNA in many normal tissues but at variable levels depending on the cell type [Stride *et al.*, 1996]. It was found at high levels in the heart, skeletal muscle, bronchial epithelial, testes and distal tubule of the kidney. MRP4 and MRP5 mRNA has been found just as widespread as MRP1 mRNA throughout the

body, while MRP2, MRP3 and MRP6 mainly occur in the liver, kidney and intestine [Borst *et al.*, 1999].

1.5.2.3 Substrates

Drug uptake experiments using inside-out membrane vesicles prepared from NIH 3T3 cells expressing MRP found a range of MRP substrates [Paul *et al.*, 1996]. Substrates included both the neutral and mildly cationic cytotoxic drugs (*e.g.*, daunorubicin, etoposide and vincristine) and the anionic products of glutathione conjugation (*e.g. S*-(*p*-azidophenacyl)-glutathione).

1.5.2.4 Summary of MRP

As has been previously seen, p-glycoprotein has a broad range of substrates however it did not transport anionic compounds or conjugated compounds, which were known to be transported. Studies led to the identification of the MRP family of transporters (MRP1 – MRP6) located throughout the body. MRP was found to be a transporter of compounds that p-glycoprotein did not transport and also some compounds that p-glycoprotein did transport. Compounds previously thought to be p-glycoprotein substrates may also or instead be MRP substrates.

1.5.3 Organic Anion-Transporting Polypeptides (OATP)

The members of the organic anion-transporting polypeptides (OATP) are another family of proteins responsible for the membrane transport of xenobiotics. However, much less is known about the OATP family than the p-glycoprotein or MRP families. Rat Oatp1 was the first to be identified and was shown to be a sodium-independent organic anion transporter [Jacquemin *et al.*, 1994]. Since then at least six OATPs have been found in rodents and eight in humans [Tirona & Kim, 2002]. Human OATP is a glycoprotein of 80 kDa, containing 670 amino-acids, from a gene localized to chromosome 12 [Lecureur *et al.*, 2000].

1.5.3.1 Mechanism of action

Most research has focused on the role of OATP in the liver where they transport bile acids rather than their role in the intestine. The mechanism of action of OATP is currently

unclear. Studies have shown that rat Oatp (the rat equivalent of the human protein) is associated with the transport of bicarbonate [Satlin *et al.*, 1997] and glutathione [Li *et al.*, 1998].

1.5.3.2 Tissue distribution / location

OATPs are expressed in many tissues including the liver, kidney, brain and small intestine [Tirona & Kim, 2002]. In the liver, all the OATPs are found in the basolateral membrane and transport solutes from blood to bile. However, the rat Oatp1 is found in the apical surface of renal proximal tubules and rat Oatp3 is found in the apical membrane of enterocytes in the intestine.

1.5.3.3 Substrates

Endogenous substrates of the OATPs include thyroid hormones, bilirubin, prostaglandins, bile acids, steroids and cholecystokinin [Tirona & Kim, 2002]. Human and rat OATPs mediated the cellular uptake of fexofenadine in a recombinant vaccinia expression system [Cvetkovic *et al.*, 1999]. The OATPs are capable of transporting organic anions, cations, and neutral or zwitterionic (molecules that have positive and negative charges that cancel each other out) compounds. These substrates show that the title Organic Anion Transporting Polypeptides is misleading as it transports more than organic anions.

1.5.3.4 Summary of OATP

There is not a huge amount of information known about the organic anion-transporting polypeptide compared to other transporters such as p-glycoprotein and MRP. Most research has focused on the role of the transporter in the liver, where it transports anions, cations and neutral compounds. The wide range of substrates makes OATP an important transporter but more research needs to be performed, especially on the role of OATP in the intestine.

1.5.4 Differences between efflux transporters

As has been implied previously, p-glycoprotein and MRP have overlapping but distinct substrates and inhibitors, while OATP has substrates overlapping the other two transporters.

Two of the transporters, p-glycoprotein and MRP1, are ABC-transporters yet only share 15% of their amino-acid sequence [Cole *et al.*, 1992]. OATP is not an ABC-transporter so has a different sequence.

The role of OATP and p-glycoprotein on the uptake and excretion of fexofenadine was studied by a number of techniques [Cvetkovic *et al.*, 1999]. Both p-glycoprotein and OATP were found to be transporters of fexofenadine. Inhibitors of p-glycoprotein (*e.g.* HIV protease inhibitors, and statins) were also found to be inhibitors of OATP. This suggests that the combined inhibition of p-glycoprotein and OATP may account for drug interactions involving fexofenadine. However, the study did not prove that OATP and p-glycoprotein are co-located on the apical surface.

In summary, p-glycoprotein and MRP are structurally related but with differing substrates and inhibitors (although some do overlap), while p-glycoprotein and OATP are structurally unrelated but with similar substrates and inhibitors.

1.5.5 Synergism between CYP3A4 and p-glycoprotein

In addition to their shared location in the small intestine, p-glycoprotein and CYP3A share a large number of substrates and inhibitors and are induced by many of the same compounds (see Table 1.3). Common substrates include dexamethasone and common inhibitors include verapamil. Transport of metabolites by intestinal p-glycoprotein is consistent with a concerted CYP3A-p-glycoprotein barrier to drug absorption. CYP3A metabolises compounds to metabolites that are eliminated by p-glycoprotein, and the metabolites are less likely to be passively reabsorbed due to their increased polarity. An example is cyclosporin, which is metabolised into many compounds that are actively transported out of the cell *via* P-glycoprotein [Benet *et al.*, 1996].

Table 1.3 The effect of compounds on CYP3A4 and p-glycoprotein

	CYP3A4		p-Glycoprotein		References
	Substrate	Inhibitor	Substrate	Inhibitor	
Calcium Channel Blockers					
Amlodipine		YES/NO		YES	A, B
Diltiazem	YES	YES	YES	YES	A, C, D, E
Felodipine		YES		YES	A, B, E
Nicardipine	YES	YES	YES	YES	A, B, D, E, F
Nifedipine	YES	YES/NO		YES	A, B, C, D, E, F
Nimodipine				YES	F
Nisoldipine		NO			B
Nitrendipine		YES		YES	B, E, F
Pranidipine	YES				G
Verapamil	YES	YES	YES	YES	A, C, D, E, F
HMG-CoA Reductase Inhibitors					
Atorvastatin		YES	YES	YES	E, H
Lovastatin	YES	YES		YES	C, H
Pravastatin		NO		NO	H
Simvastatin		YES		YES	H
Benzodiazepine anxiolytic					
Diazepam	YES				C
Midazolam	YES	YES		YES	C, D, E
Triazolam	YES				C
Histamine receptor antagonist					
Fexofenadine			YES		E
Terfenadine	YES			YES	C, E
HIV protease inhibitors					
Indinavir	YES	YES	YES		D, E
Saquinavir	YES	YES	YES		D, E
Potassium channel blocker					
Quinidine	YES	YES	YES	YES	C, D, E
Quinine				YES	E

Table 1.3 The effect of compounds on CYP3A4 and p-glycoprotein (continued)

	CYP3A4		p-Glycoprotein		Reference
	Substrate	Inhibitor	Substrate	Inhibitor	
Other compounds					
Buspirone	YES				I
Caffeine	YES				C
Carbamazepine	YES				C
Cisapride	YES		NO	YES	J, K
Clarithromycin				YES	E
cyclosporin A	YES	YES	YES	YES	C, D, E
Digoxin			YES		E
17-α-Ethinyl oestradiol	YES	YES			C, D
17-β-Estradiol	YES				C
Itraconazole		YES	YES	YES	D, E, I
Losartan			YES		E
Methylprednisolone			YES		E
Phenytoin			YES		E
Praziquantel	YES				L
Theophylline	YES				C

Source references:

A – Ma *et al.*, 2000
B – Katoh *et al.*,2000
C – Spatzenegger & Jaeger, 1995
D – Thummel & Wilkinson, 1998
E - Matheny *et al.*, 2001
F - Pascaud *et al.*, 1998
G - Kudo *et al.*, 1999
H – Wang *et al.*, 2001
I - Kivisto *et al.*, 1997
J – Abdel-Rahman *et al.*, 2000
K - Kearns *et al.*, 2002
L- Giorgi *et al.*, 2001

1.5.6 Summary of intestinal efflux transporters as a barrier to absorption

The development of intestinal multidrug resistance in cancer chemotherapy led to the identification of p-glycoprotein, a plasma membrane ATP-dependent transporter with a very broad specificity for substrates. This broad range implied a huge potential for drug-drug interactions. Further studies on resistant cell lines that did not express p-glycoprotein found a related family of transporters, the multidrug resistance protein (MRP) family. A third family of transporters, the organic anion-transporting polypeptides (OATP) have also

been identified, although significantly less research has been performed on this family compared to the other two.

All three families are expressed at various levels throughout the body and are expressed in the intestine. All three transporters have overlapping substrates and inhibitors as well as their own specific modulators. Two of the transporters, p-glycoprotein and MRP are structurally related but with differing substrates and inhibitors, while p-glycoprotein and OATP are structurally unrelated but with similar substrates and inhibitors.

An important barrier to intestinal absorption is the synergism between CYP3A4 and p-glycoprotein. Not only are they both located in the epithelial cells of the small intestine, they also share substrates and inhibitors. A number of the products of compounds metabolised by CYP3A4 are substrates of p-glycoprotein so the two proteins form a significant barrier.

1.6 *In situ* and *in vivo* models of drug absorption

In general, two types of models were available for studies of drug absorption into the blood, isolating the intestine *in situ* or *in vitro*. The animal models were based on the *in situ* isolation of intestinal loops, everted sacs, or vascularly perfused intestine. The drug was administered into the loop and the disappearance rate from the loop and/or the appearance in the blood was measured. In the alternative *in vitro* models, an intestinal segment was isolated and mounted in an Ussing chamber. In this case, the intestinal segment was used as a semipermeable membrane between a donor and receiver chamber. These models made it possible to characterise several factors that determine the rate of transepithelial drug transport [Artursson, 1989].

The models do have some drawbacks. First, they are not of human origin. Second, only a limited number of experiments can be performed on them. Third, they have a limited duration of a few hours [Artursson, 1989].

To solve these problems, new approaches were developed. These included the use of liposomes and cell culture models (See section 1.7). Enterocytes could be cultivated on permeable membranes. A large number of experiments could be performed simultaneously and over a relatively long time period. Sampling on the basolateral side of

the epithelium was possible and crossover studies could be performed. Many cell lines were tested but Caco-2 cells were the most useful [Artursson, 1989].

Although there has been a long history of research on p-glycoprotein, many questions remain. The sheer range of known substrates implies that there are a large number of compounds that are currently unknown substrates of the transporter. A quick and simple assay for inhibitors of p-glycoprotein would be useful.

1.7 Caco-2 cells

1.7.1 The use of Caco-2 cells

Caco-2 cells were first cultivated in 1977 [Fogh *et al.*, 1977] but only became widely available in the 1980s. The evolution of cells grown on a filter is generally characterised by the sequence of proliferation, confluency, and differentiation.

Among over 20 human colon carcinoma cell lines tested, Caco-2 cells were the only ones to differentiate spontaneously as normal enterocytes do. After seeding, proliferation started after a time lag of 48 hours, and confluency was reached after 5 days, but the proliferation continued until a plateau was reached after 9 days [Delie & Rubas, 1997].

During the cell culture process, cells are maintained in flasks, where they grow until confluency, at which point they are detached from the flasks and reseeded. Each of these cycles is called a passage. Cell characteristics vary from passage to passage. At a high passage number, cells are more likely to be phenotypically different from the parental cells [Delie & Rubas, 1997].

Caco-2 cells are a heterogeneous mix of subtypes, which is one reason why exact experimental results are hard to replicate from one lab to another. It is thought that passaging will select the fast growing subpopulations and so cell transport parameters could change due to this process. Caco-2 cells at low (28-36) and high (93-108) passage numbers were used to study the transport of a number of drugs [Yu *et al.*, 1997]. It was shown that passaging reduced the functional expression of a brush-border enzyme (alkaline phosphatase) and several transport proteins, reduced the morphological heterogeneity, and resulted in a higher transepithelial resistance. It can be concluded that passaging significantly affects the biological characteristics and transport properties of Caco-2 cell

monolayers. This will affect the extrapolation of experimental transport studies to make *in vivo* predictions of bioavailability.

1.7.2 Cell development over time

During differentiation, Caco-2 monolayers, grown on plastic, display domes, characteristic of transepithelial ionic transport. The number of domes reaches a peak at day 8 post seeding and then falls. Dome formation results from the accumulation of secreted compounds in the basolateral domain [Delie & Rubas, 1997].

After differentiation, Caco-2 cells are very similar to normal enterocytes with regard to their morphology. At confluency, half the cells form a thick brush-border layer while the rest form clusters. Cell dimensions vary from report to report showing the heterogeneity of Caco-2 cells, but in general they have dimensions of 16μm by 1.4μm [Delie & Rubas, 1997].

1.7.3 Correlation of Caco-2 studies with in vivo *studies*

The value of the Caco-2 cell model depends on how well it predicts permeability across the various intestinal tissues. A large number of compounds with different structural properties have been used to determine how good the correlation is [Artursson & Karlsson, 1991]. The permeability constants from Caco-2 cells were plotted against the percentage absorption in humans after oral absorption, and a good correlation was found. Compounds tested included testosterone, warfarin, acetylsalicylic acid, mannitol, and polyethyleneglycol. It was also concluded that many incompletely absorbed compounds used the paracellular route and so Caco-2 cells are a good model for drug transport *via* both routes although Caco-2 cells have tighter junctions and therefore lower paracellular transport compared to the gastrointestinal tract.

The first reports on the correlation between Caco-2 cell permeability and drug absorption in humans, led to huge generalisations that Caco-2 cell permeability was the only factor needed for *in vivo* predictions. The effects of drug solubility, liver metabolism and other factors were ignored. The effects of drug metabolising enzymes were extrapolated from small data sets with the enzymes expressed at levels different to known *in vivo* level. Some of these problems have since been solved by the development of cell lines with specific enzymes being permanently induced [Artursson & Borchardt, 1997].

1.7.4 Enzymes expressed by Caco-2 cells

Caco-2 cells are known to express a large number of enzymes and transporters (see Figure 1.2). The expression by Caco-2 cells, of enzymes mentioned in previous sections, is investigated below.

The expression of cell surface peptidases on Caco-2 cells was examined using enzyme assays [Howell *et al.*, 1992]. Eight peptidases were identified: aminopeptidase N, dipeptidyl peptidase IV, peptidyl dipeptidase A (angiotensin-converting enzyme), aminopeptidase P, aminopeptidase W, endopeptidase-24.11, γ-glutamyl transpeptidase and membrane transpeptidase. These peptidases have varying specificities of peptide substrates but work together to create the peptidase activity on the surface of Caco-2 cells.

The esterase activity of a number of homogenates from various intestinal segments and species was investigated for future studies on the effects of ester prodrugs [Van Gelder *et al.*, 2000]. There was a site-specific (duodenum > jejunum > ileum > colon) and species-specific (rat > man > pig) degradation of the substrates used. Homogenates from Caco-2 cells caused a reduced level of degradation compared to homogenates from human ileum. The results showed that Caco-2 cells do have endogenous esterase activity, albeit at a lower level than *in vivo*.

Since differentiated Caco-2 cells express various cytochrome P450 isoforms and phase II enzymes, such as UDP-glucuronyltransferases, sulfotransferases and glutathione-S-transferases, the Caco-2 model can also be used to study presystemic drug metabolism [Meunier *et al.*, 1995].

Caco-2 cells express CYP3A4 at very low levels (7.9 pmol/mg protein) [Schmiedlin-Ren *et al.*, 1997] compared to human jejunum (70±20 pmol/mg protein) [Watkins *et al.*, 1987]. Many methods have been used to increase CYP3A4 expression including exposure to 1α,25-dihydroxyvitamin D_3 [Schmiedlin-Ren *et al.*, 1997] and transfection with a vector containing CYP3A4 cDNA [Hu *et al.*, 1999]. Transfected cells had significantly increased levels of CYP3A4 activity but unchanged morphological and transport characteristics compared to the wild type although activity was lost during cell passage with a half-life of 3-4 weeks [Hu *et al.*, 1999].

1.7.5 Efflux transporters expressed by Caco-2 cells

Intestinal epithelial cells are the main barriers to the absorption of xenobiotics. Figure 1.2 showed the range of uptake transporters expressed by intestinal epithelial cells. It did not show any efflux transporters. The first membrane-bound efflux transporter to be identified in epithelial cells was p-glycoprotein [Shapiro & Ling, 1995], an ATP-dependent transporter with a broad range of cationic and neutral substrates. A later study using PCR and Western blot studies found both p-glycoprotein and MRP1 expressed in Caco-2 studies. Further studies using a variety of techniques (*e.g.* oestradiol as a substrate, reverse-transcriptase polymerase chain reaction, Northern blotting) found that MRP2 and MRP3 were expressed while MRP1 and MRP5 expression was minimal [Hirohashi *et al.*, 2000]. No study has investigated the expression of OATP in Caco-2 cells.

1.7.6 Summary of Caco-2 cells

Caco-2 cells have been used as a research tool for over twenty years. A mix of subtypes develop over time (as they are passaged), confusing results from lab to lab, so studies have to use cells from a similar passage number.

The permeability constants of compounds tested on Caco-2 cells correlate well with *in vivo* studies. Other factors, such as drug solubility and liver metabolism, need to be taken into account before *in vivo* predictions can be made.

Caco-2 cells express a number of intestinal enzymes and transporters. These include peptidases and esterases utilised by prodrugs as well as metabolising enzymes. There have been shown to express both p-glycoprotein and MRP but not yet OATP. The level of expression varies from subtype to subtype but cell lines can be induced to express certain proteins at a high level.

The use of Caco-2 cells as an *in vitro* model for drug absorption has a long history. They can be used to model the uptake of peptides and peptide prodrugs, as well as model the transport of compounds across a cell monolayer.

1.8 The effect of natural compounds on the pharmacokinetics of co-ingested drugs

Pharmacokinetics is the study of the rates at which a drug is absorbed, distributed around the body, metabolised, and eliminated from the body. This encompasses a large number of processes that can be modulated by other compounds. As natural compounds have been consumed since life began for food and medication, it is not surprising that some of them have the potential to alter the pharmacokinetics of drugs.

1.8.1 The effect of St. John's Wort on drug absorption

St. John's Wort (*Hypericum perforatum*) is an over-the-counter herbal remedy used for the treatment of depression, anxiety, and sleep disorders. A repeated dose of St. John's Wort (300 mg three times a day for 14 days) significantly increased the urinary ratio of 6-β-hydroxycortisol and cortisol [Roby *et al.*, 2000]. It was concluded that St. John's Wort was an inducer of CYP3A4. A later study [Wang *et al.*, 2001b] compared the effects of short-term dosing (900 mg) and long-term dosing (300 mg three times a day for 14 days) on the kinetics of a number of drugs used as markers for the activities of a number of cytochrome P450 isoforms. Both treatment protocols reduced the AUC and C_{max} (these pharmacokinetic parameters are explained in Section A4.1) of orally administered midazolam although only chronic treatment caused a significant effect. There was no change in the *in vivo* activity of CYP1A2, CYP2C9 or CYP2D6. It was concluded that St. John's Wort solely induced CYP3A4.

As CYP3A is associated with p-glycoprotein, it was hypothesised that St. John's Wort may also induce p-glycoprotein expression and this was investigated by Hennessy *et al.* [2002]. The expression of p-glycoprotein was increased 4.2-fold in treated subjects.

This means that St. John's Wort induces both CYP3A4 and p-glycoprotein and may affect the pharmacokinetics of all the CYP3A substrates listed in Table 1.1 and p-glycoprotein substrates listed in Table 1.2. As it is a widely consumed remedy, there has been a publicity campaign by the UK and US governments to raise awareness of potential herb-drug interactions.

1.8.2 The effect of garlic on drug absorption

Fresh garlic (*Allium sativum*) can be processed into a number of commercial formulations including, being macerated into vegetable oils and being powdered. There has been concern about the possibility of garlic-drug interactions due to the active nature of garlic. The beneficial properties of garlic preparations include antimicrobial and antihypertensive activities.

Early studies found that garlic oil suppressed the induction of rat hepatic CYP2E1 and induction of glutathione-S-transferase and microsomal epoxide hydrolase activity [Kwak *et al.*, 1995]. Aqueous extracts of garlic also inhibited the activity of NADPH-cytochrome P450 reductase and NADH-cytochrome b5 reductase activity in porcine hepatic microsomes [Oelkers *et al.*, 1992].

Two HIV-positive patients taking ritonavir experienced serious dose-dependent adverse events after taking either fresh garlic or a garlic supplement [Laroche *et al.*, 1998]. Further studies found that fresh garlic and a garlic supplement both produced a dose-dependent inhibition of the formation of 6β-hydroxytestosterone using microsomes containing human CYP3A4 [Foster *et al.*, 1998]. A later clinical study on the effect of the garlic supplement on ritonavir kinetics found that the garlic caused a non-significant decrease in AUC and a higher clearance of the drug [Choudri *et al.*, 2000].

The effect of garlic and a number of garlic products on a range of human cytochrome P450 isozymes and p-glycoprotein was studied using an *in vitro* assay and marker substrates [Foster *et al.*, 2001]. The garlic and garlic products inhibited the activity of CYP2C9*1, CYP2C19, CYP3A4, CYP3A5 and CYP3A7, stimulated the activity of CYP2C9*2 and had no effect on the activity of CYP2D6. Investigating the effect of garlic on the ATPase activity of recombinant human p-glycoprotein membranes found that Aged, Chinese and Common garlic extracts were all stimulators, although they were not as potent as 20 μM verapamil. Elephant garlic extract had no significant effect on the ATPase activity. Stimulation of ATPase activity has been correlated with increased inhibition of p-glycoprotein. These results suggest that some (currently unknown) garlic constituents are inhibitors of CYP2C, CYP2D, CYP3A and p-glycoprotein.

The consumer awareness of the potential effects of garlic is probably much more limited than the potential effects of St. John's Wort. This may be due to the perception that St. John's Wort is seen as a medicine while garlic tablets are seen as more like vitamins.

1.8.3 The effect of co-administration of grapefruit juice on pharmacokinetic parameters of drugs

In 1991, grapefruit juice was shown to significantly increase the oral availability of the drug felodipine [Bailey *et al.* 1991]. Since then, the number of drugs recognised to be affected by grapefruit juice has grown enormously. Most of these drugs are metabolised by CYP3A4 and it was assumed that the mechanism of action was *via* inhibiting CYP3A4. Grapefruit juice did not influence the clearance of CYP3A4 substrates when they were administered intravenously [Ducharme *et al.*, 1995], it did not alter liver CYP3A4 activity, intestinal expression of CYP1A1 or CYP2D6 but did reduce intestinal expression of CYP3A4 [Lown *et al.*, 1997]. Another study found that there was a 60% down-regulation of CYP3A4 protein levels in the small intestine following repetitive daily grapefruit juice consumption [Schmiedlin-Ren *et al.*, 1997]. As the corresponding CYP3A4 mRNA level stays the same, it was concluded the grapefruit juice constituents caused a rapid degradation of the CYP3A4 protein.

Table 1.3 above listed a number of drugs and indicated whether they were substrates or inhibitors of CYP3A4 or p-glycoprotein. Appendix A2 is a summary of all the clinical trials performed to determine the effect of grapefruit juice on three pharmacokinetic parameters for a number of drugs. Table 1.4 below summarises the studies for each compound. Although grapefruit juice increases the area under the systemic concentration-time curve (AUC) for most of the drugs, the magnitude and significance of the AUC increase was highly variable. In general, if the drug is a substrate for CYP3A4 and p-glycoprotein, then consumption of grapefruit juice normally causes a significant increase in AUC and C_{max}, although the effect on t_{max} was less clear. This is not unexpected as inhibiting the metabolism and efflux of the drug will slow the movement of the drug out of the blood, increasing the amount of drug in the blood stream and the duration of its stay there. The lack of a significant effect on t_{max} may have been due to the units used (generally hours) and therefore the low value of this parameter (<2) and the size of change needed to declare a significant change. It may also have just been less sensitive than C_{max} and AUC.

Table 1.4 The effect of grapefruit juice on drug pharmacokinetics

	Effect on parameter [a]			Substrate / Inhibitor [e]	
	AUC [b]	C_{max} [c]	t_{max} [d]	CYP3A4	p-glycoprotein
Calcium Channel Blockers					
Amlodipine	↑/NO	↑/NO	NO	YES/NO	YES
Diltiazem				YES	YES
Felodipine	↑	↑	NO	YES	YES
Nicardipine	↑			YES	YES
Nifedipine	NO	NO	NO	YES/NO	YES
Nimodipine	↑	↑	↑		YES
Nisoldipine	↑	↑	NO	NO	
Nitrendipine	↑	↑	NO	YES	YES
Pranidipine				YES	
Verapamil	↑	NO	NO	YES	YES
HMG-CoA Reductase Inhibitors					
Atorvastatin	↑	NO	↑	YES	YES
Lovastatin	↑	↑	NO	YES	YES
Pravastatin	NO	NO	NO	NO	NO
Simvastatin	↑	↑	NO	YES	YES
Benzodiazepine anxiolytic					
Diazepam	↑	↑	↑	YES	
Midazolam	↑	↑	NO	YES	YES
Triazolam	↑	↑	NO	YES	
Histamine receptor antagonist					
Fexofenadine	↓	↓	NO		NO
Terfenadine	↑	↑	↑	YES	YES
HIV protease inhibitors					
Indinavir	NO	NO	↑	YES	YES
Saquinavir				YES	YES
Potassium channel blocker					
Quinidine	NO	NO	↑/NO	YES	YES
Quinine	NO	NO	NO		YES

Table 1.4 The effect of grapefruit juice on drug pharmacokinetics (continued)

	Effect on parameter [a]			Substrate / Inhibitor [e]	
	AUC [b]	C_{max} [c]	t_{max} [d]	CYP3A4	p-glycoprotein
Other compounds					
Artemether	↑	↑	↓		
Buspirone	↑	↑	↑	YES	
Caffeine				YES	
Carbamazepine	↑	↑	NO	YES	
Cisapride	↑	↑	↑	YES	YES
Clarithromycin	NO	NO	↑		YES
cyclosporin A	↑	↑	NO	YES	YES
Digoxin	↑	NO	NO		YES
17-α-Ethinyl oestradiol	↑	↑	NO	YES	
17-β-Estradiol				YES	
Itraconazole	NO	NO	NO	YES	YES
Losartan	NO				YES
Methylprednisolone	↑	↑	↑		YES
Phenytoin					YES
Praziquantel	↑	↑	NO	YES	
Theophylline	NO	NO	NO	YES	

Key:

[a] Direction of any significant effect of grapefruit juice on pharmacokinetic parameter, summarised from Table A.4

[b] Area under the curve of blood concentration against time

[c] Maximum blood concentration reached

[d] Time to maximum blood concentration

[e] Either a substrate or and inhibitor of the two proteins, data from Table 1.3

The calcium channel blockers follow the general trend. Clinical studies on the effect of grapefruit juice on amlodipine kinetics give mixed results. One study found a statistically significant increase in AUC and C_{max} (~14% increase) while another study did not (~7% increase). The grapefruit juice obviously had an effect, the question is whether it was clinically significant or not. This question will not be resolved until more studies are performed on this drug. Mixed results were also found for many of this family of compounds.

The studies on the effect of the juice on felodipine (over 20) show the very significant ($p<0.01$) increase in AUC and C_{max} (~12-300% increase). They also show the effect of the timing of the consumption of grapefruit juice. Drinking juice three times a day for five days leading up to the administration of the drug resulted in a significant 211% increase in AUC ($p<0.001$) and significant 334% increase in C_{max} ($p<0.001$) [Lown *et al.*, 1997]. Drinking one glass at the same time as taking the drug resulted in a significant ($p<0.001$) 92% increase in AUC and 145% increase ($p<0.001$) in C_{max} [Bailey *et al.*, 1996]. Even one glass (200ml) consumed 24 hours before the administration of the drug caused a non-significant ($p>0.05$) 12% increase in AUC and a significant ($p<0.05$) 32% increase in C_{max} [Lundahl *et al.*, 1995]. Although the largest increases were found after repeated consumption of grapefruit juice, significant effects were measured after co-consumption of the juice and the drug.

A number of studies have been performed on nifedipine with mixed results. Grapefruit juice causes a small increase in the parameters for nifedipine, but like amlodipine this is only sometimes significant. This may be related to the weak ability of nifedipine to inhibit CYP3A4 and hence be affected by that enzyme.

The effect of grapefruit juice on the family of compounds that are HMG-CoA reductase inhibitors ("the statins") is clearly related to the compounds ability to inhibit CYP3A4 and p-glycoprotein. Atorvastatin, lovastatin and simvastatin are inhibitors and are affected. Pravastatin is not an inhibitor and not affected by grapefruit juice, and therefore stands out as an effective medicine that will have fewer interactions than the other members of the family.

The benzodiazepine anxiolytic drugs follow the general trend but the histamine receptor antagonists are affected in opposing ways by the juice. Grapefruit juice (300ml), consumed at the same time as fexofenadine (120mg), caused a very significant ($p<0.01$) 67% decrease in AUC, and a very significant ($p<0.01$) 62% decrease in C_{max} [Dresser *et al.*, 2002]. Grapefruit juice (300ml), consumed half an hour before consuming terfenadine (120mg), caused a significant ($p<0.05$) 149% increase in AUC, and a significant ($p<0.05$) 243% increase in C_{max} [Clifford *et al.*, 1997]. Although there have been a number of studies of the effect of the juice on terfenadine, there has only been one study of its effects

on fexofenadine. As both drugs are inhibitors of p-glycoprotein, the divergent effects definitely imply that more clinical studies should be performed.

Two HIV protease inhibitors have had the effect of grapefruit juice tested on them. In the case of indinavir, the juice did not have a significant effect on AUC or C_{max} but did significantly increase t_{max} [Shelton *et al.*, 2001]. However, the consumption of 150ml of juice with saquinavir caused a 40% increase in AUC and 60% increase in C_{max}, while 300ml of the juice caused a 120% increase in both AUC and C_{max} [Kupferschmidt *et al.*, 1998]. Yet again more studies should be performed but it can be concluded that the juice at least affects saquinavir.

Most of the remaining compounds follow the previously discussed trend of an increase in AUC and C_{max}. The exceptions include quinidine and quinine, clarithromycin, itraconazole, losartan and theophylline. In those cases, the drugs are known inhibitors of p-glycoprotein. The weaker inhibitors of the transporter may only be weakly affected by the co-consumption of the juice and therefore no significant effect may be seen.

1.8.4 Summary of the effect of natural compounds on drug metabolism

After they have been absorbed, drugs undergo a number of processes included metabolism and excretion. These processes can be modulated by the consumption of various naturally occurring products. One of the more widely known interactions is between St. John's Wort (a herbal antidepressant) and many drugs that are substrates of CYP3A4 and p-glycoprotein. St. John's Wort induces both proteins and can seriously affect the metabolism of a number of drugs. Garlic formulations have been found to inhibit the activity of both proteins, and can cause just as serious interactions as St. John's Wort. In 1991, it was discovered that the consumption of grapefruit juice significantly increased the oral availability of felodipine [Bailey *et al.*, 1991]. Since then a large number of clinical studies have examined the effect of grapefruit juice on the pharmacokinetics of a wide selection of drugs. In general, if the drug is a substrate for CYP3A4 or p-glycoprotein then it is likely that consumption of the juice will cause significant effects on the drugs kinetics, potentially creating a clinically significant interaction. The variations in the magnitude of the effect of the juice on the kinetics of the drug, is related to variations in the drugs ability to inhibit the proteins. Grapefruit juice obviously contains natural compounds that are significantly strong inhibitors of the proteins. If a natural inhibitor of p-glycoprotein could

be isolated from the juice, it could be given with a drug that is a p-glycoprotein substrate to increase the absorption of that drug.

1.9 Aim of Thesis

The aim of this thesis is to study mechanistic aspects of absorption and efflux to inform drug design and *in vitro* testing. The first part will investigate increasing drug uptake across the cell membrane into the cell. The second part will investigate drug efflux back into the intestine and identify ways of reducing it. Combined together, the two parts will show a number of methods of increasing drug absorption from the intestine to the blood.

1.9.1 Investigating an uptake transporter

This part of the thesis will investigate the dipeptide transporter and structural features which may enhance drug transport *via* the transporter. One objective of this thesis will be to identify structure-activity relationships for the intestinal dipeptide transporter, by developing a rapid screening system and using it to determine the affinities of a number of dipeptide derivatives. The result will be the identification of an optimum substrate for the transporter, and therefore inform prodrug design.

1.9.2 Investigating efflux transporters

This part of the thesis will investigate p-glycoprotein as the main intestinal efflux transporter. The first objective will be to develop an *in vitro* assay for the transporter. The second objective will be to validate it by determining the effect of known inhibitors of p-glycoprotein, compounds that are known to have no significant effects on the transporter and inhibitors of MRP. The final objective will be to identify the effect of grapefruit juice and other natural compounds on p-glycoprotein. The result will be the identification of an assay for the screening of compounds that may inhibit p-glycoprotein and also the identification of safe natural compounds that can be co-consumed with a drug to increase the absorption of the drug.

2. GENERAL MATERIALS AND METHODS

ABSTRACT:

The general materials and methods used routinely for the work of this thesis are presented here. Supplementary and modified experimental procedures are described in the relevant sections of subsequent chapters.

2.1 Materials

2.1.1 Cell culture

Caco-2 cells were supplied from the American Type Tissue Culture Collection, Rockville, MD, USA. Dulbecco's Modified Eagle Medium (DMEM) (with sodium pyruvate and glutamine cat. 41966-029), Foetal Bovine Serum, N-2 hydroxyproperazine–N'-2 ethanosulphonic acid (HEPES) buffer, glutamine, non-essential amino acids, penicillin / streptomycin and trypsin were supplied by Gibco. Phosphate-buffered-saline (PBS), EDTA, trypan blue and proline were supplied by Sigma. Plastic flasks were supplied by Costar. Cells were incubated in a Jencons Millenium incubator and counted using haemocytometer from Appleton Woods.

2.1.2 Liquid scintillation counting

Optiphase HiSafe III scintillant was supplied from LKB, UK. The scintillation counter was a Hewlett Packard Tricard 2000 CA liquid scintillation analyser.

2.1.3 Protein determination

The BCA protein assay containing copper sulphate pentahydrate (4% w/v) and BCA reagent solution, and bovine serum albumin was supplied by Sigma. A Jenway 6105 spectrophotometer was used to measure absorption.

2.1.4 Radioactive uptake studies

[^{3}H]Gly-Pro was supplied from Dupont New England Nuclear (NEN) Research Products, Boston, MA, USA. Hank's Balanced Salt Solution (HBSS), 2-[N-morpholino]ethanesulphonic acid (MES) buffer, proline, PBS, sodium hydroxide, sodium azide, Triton X-100, DMSO, dipeptides, and amino acids were supplied by Sigma. The HEPES buffer was supplied by Gibco. The 24-well plates were supplied by Costar.

2.1.5 Radioactive transport studies

[^{3}H]-vincristine sulphate was obtained from Amersham Life Sciences Ltd. The [^{14}C]-mannitol, Hank's Balanced Salt Solution (HBSS) and sodium hydroxide was supplied by Sigma. The six well inserts and plates were supplied by Costar.

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Culture Media

Culture medium was used in culturing Caco-2 in plastic flasks and plates. It comprised of Dulbecco's Modified Eagle Medium (DMEM), with 10% Foetal Bovine Serum, 0.5% (HEPES) buffer, 1% L-glutamine, 1% Non-Essential Amino Acids (NEAA), 1% Penicillin/Streptomycin (100 U/ml). The medium was stored at 4°C and replaced after two weeks.

2.2.1.2.Cell Stock solution

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 75 cm^{2}. The cells were grown as monolayers at 37°C in 5% carbon dioxide and 90% relative humidity. Every other day, the medium in the flasks (or wells) was aspirated and replaced.

When the cells in a flask reached confluency (after around 5 days), they were passaged to create a stock solution which was then used to seed new flasks or plates for experiments. The culture medium in the flask was aspirated and the cells were washed with pre-warmed PBS/EDTA (1 x 5ml x 4 minutes) before the solution was aspirated. The cells were then washed with trypsin/EDTA (1 x 5ml x minute), the solution was aspirated, and the cells were incubated for 8 minutes. Fresh culture medium (10ml) was added and washed over the cells a number of times to create a single-cell-suspension. The single-cell-suspension (10ml) was used to seed three flasks. The cell suspension (1ml) was added to fresh culture media (11ml) in each new flask. The flasks were then left in the incubator for the cells to grow.

2.2.1.3 24 Trypan blue exclusion test for viable-cell density

In order to check the viable-cell density, a trypan-blue exclusion test was performed when seeding cells onto plates and inserts. A 10-fold dilution of the suspension (0.5ml suspension in 4.5ml fresh media) was used. Trypan blue (100µl) was added to the diluted suspension (400µl, 1.25 fold dilution). The mixture was used to count cells on a haemocytometer. The haemocytometer had five squares visible under a light microscope. Viable cells appeared with a clear cytoplasm, while dead cells appeared blue. The number of cells per ml of cell suspension could be calculated using the following formula.

Equation 2.1 Calculation of cell concentration

$$Cells/ml = \left(\frac{CellsCounted}{5}\right) * 1.25 * 10^5$$

Cells suspensions where viable cells accounted for less than 95% were discarded.

2.2.2 Liquid scintillation counting

Beta-emitting radionuclides [^{3}H] and [^{14}C] were quantified using liquid scintillation spectrophotometry. Optiphase Hisafe III (5ml) was added to every sample and mixed before being counted on a Hewlett Packard Tricard 2000 CA liquid scintillation analyser (5 minutes per sample). In all cases counts per minute (CPM) were converted into disintegrations per minute (DPM) by comparison with standard quench-correction curves.

2.2.3 Protein determination

The protein content of cell monolayers was determined using a BCA protein assay kit. The protein reduces copper from Cu^{2+} to Cu^{1+} in a concentration-dependent manner. Bicinchoninic acid (BCA) is a chromogenic reagent for Cu^{1+}, producing a purple complex with a maximum absorbance at 562nm.

The protein determination reagent was produced by combining one part copper (II) sulphate pentahydrate (4% w/v) with 50 parts BCA reagent solution. Bovine serum albumin (0-100µg) was tested to create a calibration curve. The sample to be tested (100µl of protein standard or unknown) was mixed with the protein determination reagent

(1900µl), incubated at 37°C for an hour, and the absorbance at 562nm was read using a spectrophotometer. A calibration curve of absorbance vs. protein concentration was then used to calculate the amount of protein in the unknown samples.

2.2.4 Radioactive uptake experiments

2.2.4.1 Solutions

The uptake experiments required four solutions: Transport medium (pH 6), Gly-Pro solution, stop solution, and detergent solution.

Transport medium (pH 6) contained Hank's Balanced Salt Solution (HBSS) (9.76 g L^{-1}), MES buffer (25mM), proline (10mM) and made to volume with double-distilled water. The solution was adjusted to pH 6 using sodium hydroxide (1M) and stored at 4°C.

The Gly-Pro solution is the most important solution. It contains the radiolabel as well as any compounds being tested. This solution is the transport media with a few additions. When testing the inhibitory effects of compounds, 2ml of Gly-Pro solution containing each inhibitor or control was needed (0.5ml/well x 4 wells). Therefore, the compounds were dissolved in 4ml transport media (pH 6) (2ml excess) containing [^{3}H] Gly-Pro (50nM). As a number of the compounds were not very soluble, 1% DMSO was added to all solutions to help them dissolve. All compounds tested had a final concentration of 1mM.

The stop solution contained 0.25g sodium azide dissolved in 500ml PBS. This 0.05% w/v solution toxic to the cells and so stops any further uptake after the experiment has finished. The solution was stored at 4°C.

The detergent solution contains 5ml of Triton-X dissolved in 500ml double-distilled water. This 1% v/v solution is used to dissolve the cell membranes. The solution was stored at room temperature.

2.2.4.2 Basic protocol

The cells were seeded on plastic 24-well plates at a density of 8 x 10^5 cells $well^{-1}$. The culture media (1ml $well^{-1}$) was replaced on alternate days and the cells were used on the seventh day post-seeding. The transport media and Gly-Pro solutions were warmed to

37°C, while the stop solution was stored on ice. The culture media from each well was aspirated and replaced by transport media (pH 6) (0.5ml per well). After 5 minutes, the transport media was aspirated and replaced by Gly-Pro solution (0.5ml per well, four wells per condition). The radioactive solution was left on the cells for 3 minutes before being collected into a scintillation vial. The cells were then washed twice with ice-cold stop solution (0.5ml/well) for five minutes. The washings were collected in two scintillation vials. Detergent solution was then added to each well (1ml/well) and the plate was left in an incubator overnight. The resulting solublised cell solution was collected into a scintillation vial. Each vial had 5ml scintillation fluid added to it and then had its radioactivity measured by liquid scintillation counting. Appendix A3 contains the method of calculating the concentration of Gly-Pro inside the cells and IC50 values from radioactivity contained in the vials.

2.2.4.3 Effect of time on Gly-Pro uptake

The Gly-Pro solution was added to the cells for a range of times (0.5-60 minutes) instead of 3 minutes.

2.2.4.4 Effect of pH on Gly-Pro uptake

Normal transport media was adjusted to pH 5.0, 5.5, 6.0 and 6.5 using sodium hydroxide (1M). A new solution containing HEPES buffer (25mM) instead of the MES buffer was made and then adjusted to pH 7.0 and 7.5. The solution was stored at 4°C. The uptake at the six different pH values was measured.

2.2.4.5 Effect of temperature on Gly-Pro uptake

Two plates were seeded to determine the effect of temperature. The uptake of Gly-Pro at 4°C and at 37°C was then measured.

2.2.4.6 Effect of proline on Gly-Pro uptake

Caco-2 cells produce prolidase, an enzyme that cleaves Gly-Pro. The presence of this enzyme could mean that [^{3}H] proline is entering the cells not [^{3}H] Gly-Pro. Excess proline (10mM) is normally added to saturate the amino acid transporter and stop [^{3}H] proline entering the cell. The radioactivity in the cells should therefore all be Gly-Pro. Transport

media not containing proline was produced to identify the difference between the presence and absence of proline on Gly-Pro uptake.

2.2.4.7 Effect of concentration on Gly-Pro uptake

The uptake of Gly-pro at six different concentrations (0-10mM) in the presence and absence of an inhibitor (SQ29852) was measured. Appendix A1 shows how the kinetic parameters K_m and V_{max} can be derived from this experiment.

2.2.5 Transport of radioactive Vincristine studies

2.2.5.1 Solutions

The transport experiments required five solutions: Transport medium (pH 7.4), two donor solutions, stop solution, and detergent solution.

Transport medium contained Hank's Balanced Salt Solution (HBSS) (9.76 g L^{-1}), HEPES buffer (25mM) and made to volume with double-distilled water. The solution was adjusted to pH 7.4 using sodium hydroxide (1M) and stored at 4°C.

The donor solution (no inhibitor) was transport media (pH 7.4) containing [^{3}H]-vincristine (10μM) and [^{14}C]-mannitol (1μM). The donor solution (plus inhibitor) was transport media (pH 7.4) containing [^{3}H]-vincristine (10μM), [^{14}C]-mannitol (1μM), and an inhibitor (100μM).

The stop solution and detergent solution were the same as in 2.2.4.1 (solutions).

2.2.5.2 Basic protocol

The cells were seeded onto 6-well polycarbonate inserts (4 x 10^5 cells ml^{-1}, 2ml per insert). Culture media (2ml) was added to the basolateral compartment. The cells were incubated at 37°C in 5% carbon dioxide and at 90% relative humidity. The culture media was replaced on alternate days and the inserts were used 21 days post seeding.

The existing culture media was aspirated and replaced by transport media (pH 7.4) for 15 minutes, then aspirated. For apical-to-basolateral (A-B) transport, a donor solution (1.5ml)

was added to the apical compartment and transport media (pH 7.4) (2.6ml) added to the basolateral compartment. For basolateral-to-apical (B-A) transport, a donor solution (2.6ml) was added to the basolateral compartment and transport media (pH 7.4) added to the apical compartment. In each experiment there were four conditions: A-B and B-A with no inhibitor and A-B and B-A with inhibitor. There were three inserts for each condition. The plates were incubated at 37°C over the course of the experiment. Every ten minutes the plates were removed form the incubator and placed on a rotating platform to distribute the radionuclides evenly in the receiving chamber. A sample (200μl) was taken and put into a scintillation vial and immediately replaced with transport media (pH 7.4). After the last sampling point, the remaining solutions in each compartment were collected into scintillation vials. The cells were washed twice with the stop solution (2 x 2ml in each compartment) and the washings collected separately. They were then incubated overnight with the detergent solution (2ml in each compartment). The solubilised cells were collected into scintillation vials. Scintillation fluid (5ml) was added to each vial and the radioactivity determined by liquid scintillation counting. Appendix A4 contains the method of calculating the apparent permeability (Papp) (cm / mg protein / s) from the radioactivity detected in the vials.

3. INVESTIGATING DIPEPTIDE TRANSPORT: INHIBITION OF GLY-L-PRO UPTAKE

ABSTRACT:

Dipeptides can be absorbed into cells, where they are digested and excreted into the blood as amino acids. Absorption is *via* the dipeptide transporter (which also transported tripeptides and dipeptide derivatives). The optimum conditions for measuring inhibition of Gly-Pro uptake were found to be measured after three minutes, at pH 6, at 37°C and where the uptake medium contained less than 2% DMSO. A number of structure-activity relationships were identified. Increasing the amino-acid chain-length increased IC_{50} but then decreased it. The presence of a thiol group in the side-chain increased IC_{50} while the presence of a hydroxyl group did not. The methyl and benzyl esters of the dipeptides had significantly different effects on affinity indicating they were stable compounds and that they were not being hydrolysed. The benzyl esters had lower or equal IC_{50} values compared to the parent dipeptides while the methyl esters had higher values. Diphenylmethyl esters had larger IC50 values than the benzyl esters. Aspartyl and glutamyl derivatives had a range of IC_{50} values but in general, negatively charged derivatives had larger IC_{50} values than uncharged derivatives. Sarcosylproline derivatives had low IC_{50} values while the other non-dipeptide derivatives (based on dicarboxylic acids) had much larger IC_{50} values. Statistical analyses correlated IC_{50} with the heat of formation, number of hydrogen-bond acceptor atoms in the molecule, the size of the molecule, R_2 and R_3 groups and the dipole moment of the molecule. These results indicated that while molecular properties did affect IC_{50}, the size, charge and composition of the R_1, R_2 and R_3 groups caused the most significant effects, supporting the structure-activity relationships identified.

3.1 Introduction

The historical background to research into peptide absorption and the discovery of the dipeptide transporter was covered in Section 1.4 of the Introduction. Briefly, for many years it was thought that proteins had to be hydrolysed to amino acids before they could be absorbed in the intestine, however later research found that dipeptides could be absorbed into cells, where they were digested and excreted into the blood as amino acids. This led to the discovery of the dipeptide transporter (which also transported tripeptides) and its gene, hPEPt1. A number of dipeptide derivatives and peptidomimetic drugs have been found to be substrates for the transporter, including the ACE inhibitors and β-lactam antibiotics. This broad range of substrates led to the development of prodrugs that targeted the transporter. These prodrugs were developed to target the transporter; however there has been little research on the optimum structural features required by prodrugs to be transporter substrates.

3.1.1 In vitro *uptake of dipeptides*

The binding and uptake of dipeptides composed of various amino acid residues have been examined to identify structural requirements for uptake. These experiments are examined below.

The uptake of diastereomers of Val-Val and their effect on the uptake of [^{3}H]-cephalexin into Caco-2 cells was studied to see if binding to the dipeptide transporter correlated with cellular uptake [Tamura *et al.*, 1996]. It had previously been shown that the incorporation of one D-amino acid in Val-Val does not reduce affinity for the dipeptide transporter, although the presence of two does [Hidalgo *et al.*, 1995]. However, this later study showed that the incorporation of one D-amino acid significantly reduced the affinity for the transporter. The effect was more significant if it was incorporated at the C-terminal end. The difference in results may have been due to the different concentrations of Val-Val diastereomers used (10 mM by Tamura *et al.* and 20 mM used by Hidalgo *et al.*). The apical uptake of L-Val-D-Val (LD-isomer) and D-Val-L-Val (DL-isomer) were 15 and 50 times higher than that for D-Val-D-Val (DD-isomer). The uptake of the LD-isomer and DL-isomer was inhibited by Gly-Pro but the uptake of the DD-isomer was not. It was concluded that the uptake of the LD-isomer and DL-isomer are mainly carrier-mediated by the dipeptide transporter and that the uptake of the DD-isomer is mainly by passive diffusion. L-Val-L-Val (the LL-isomer) was not detected by HPLC in the Caco-2 cells.

Since it is a natural dipeptide and has the highest affinity for the dipeptide transporter among the Val-Val diastereomers, it was assumed that it is actively taken up into the cells but rapidly metabolised. It was concluded that the binding of Val-Val diastereomers to the dipeptide transporter appeared to be a good predictor of their cellular uptake.

Dipeptides containing sarcosyl residues have modified peptide bonds, with a methyl group on the bond nitrogen instead of a hydrogen atom. The relationship between the structure of substrates containing sarcosyl residues and affinityaffinity for the dipeptide transporter has been studied in kidney brush-border membrane vesicles (BBMVs) [Daniel *et al.*, 1992]. Dipeptides containing one sarcosyl residue (Gly-Sar, K_i = 0.372±0.017 mM) had a significantly worse affinity compared to dipeptides containing no sarcosyl residues (Gly-Gly, K_i = 0.091±0.005 mM). The presence of two sarcosyl residues abolished affinity for the transporter (Sar-Sar, K_i = 15.92±0.60 mM).

The affinity of charged dipeptides for the dipeptide transporter was examined by studying the inhibition of the uptake of [^{14}C]-Gly-L-Pro into brush-border membrane vesicles from whole rabbit small intestine [Wootton & Hazelwood, 1989]. It was concluded that the effects of charge on affinity are additive. A single positive charge had no effect but a negative charge or a double positive charge produced a considerable loss in affinity. The structural features of dipeptides, required for interaction with the dipeptide transporter in Caco-2 cells, were identified by studying the inhibition by dipeptides of cephalexin uptake [Hidalgo *et al.*, 1995]. Linear dipeptides, containing neutral, basic or acidic amino acid residues, inhibited [^{3}H]-cephalexin uptake by the same amount (K_i all <1mM). This contradicted previous results [Wootton & Hazelwood, 1989] but this may have been due to the difference between using BBMVs and Caco-2 cells or the substrate used in the competition studies (Gly-Pro vs. cephalexin).

Some dipeptides were cyclised to examine the role of the α-carboxyl and α-amino groups [Hidalgo *et al.*, 1995]. It was concluded that the inability of cyclised dipeptides to interact with the transporter could be overcome by the choice of amino acid residues in the dipeptide. However, later experiments led to the conclusion that cyclic dipeptides do not have any affinity for the dipeptide transporter and are transported *via* a paracellular route (around cells rather than through them).

In summary, experiments on the affinity of the dipeptides to the transporter have identified a number of optimum characteristics. These include the presence of L-amino acids, the lack of sarcosyl residues, and the lack of cyclic residues. The effect of positive and negative charges was unclear.

3.1.2 In vitro *uptake of dipeptide derivatives*

A number of dipeptide derivatives and peptidomimetic drugs have been found to be substrates of the dipeptide transporter. Studies on these compounds have also helped to identify optimal structural features for at least the binding of the substrates of the transporter.

The uptake, into rat jejunal segments, of a series of dipeptide analogues lacking the N-terminal α-amino group, were studied to see if this functional group was required for transport [Bai *et al.*, 1991]. The uptake of compounds such as phenylacetylproline and N-benzoylglycine (hippuric acid) was significantly reduced by the presence of dipeptides. It was concluded that the presence of the α-amino group is not necessary for transport *via* the dipeptide transporter. This conclusion was supported by studies using kidney BBMVs [Daniel *et al.*, 1992] that found that blocking the α-amino group with an acetyl group or *t*-butyloxycarbonyl group caused a significant ($p<0.001$) decrease in affinity.

The same series of experiments found that changing the α-carboxyl group to an amino group caused a significant ($p<0.001$) decrease in affinity, as did the creation of an ethyl ester at this point. The benzyl ester also had a decreased affinity for the transporter compared to the parent compound but better than the ethyl ester. Later experiments investigated the effect of modifying the carboxylic acid in Val-Val by esterification or reduction to the alcohol [Hidalgo *et al.*, 1995]. The alcohol (Val-Val-OH) and methyl ester (Val-Val-OMe) only had half the activity of Val-Val. It was concluded that the ability to inhibit uptake is dependent on the presence of a free α-carboxyl group.

Novel lipophilic derivatives of peptides using chemical modification with fatty acids have been shown to have significantly improved intestinal absorption in rats, compared to the parent compound, due to their increased chemical stability and increased lipophilicity (*e.g.* thyrotropin-releasing hormone [Yamada *et al.*, 1992]). Butyric and caproic acids were conjugated to the N-terminus of phenylalanylglycine (Phe-Gly) and their uptake into rabbit

intestinal BBMVs examined [Fujita *et al.*, 1997]. The uptake of the derivatives was significantly greater than the uptake of Phe-Gly and was inhibited by other dipeptides. It was concluded that the derivatives were absorbed via the dipeptide transporter and therefore that dipeptides can be modified yet still transported by the dipeptide transporter.

The effect of modifying the side-chain of one of the residues on the affinity to the dipeptide transporter was studied using esters of the β-carboxylic acid of D-Asp-Ala to inhibit the uptake of [^{14}C]-Gly-Sar [Taub *et al.*, 1997a]. Both the cyclohexyl (C_6H_{11}) and benzyl ($CH_2C_6H_6$) esters had lower IC_{50}s than the parent compound (2.80±0.11 mM and 2.62±0.35 mM vs. 5.75±0.09 mM). It was concluded that it was possible to modify side-chains and still retain affinity for the dipeptide transporter. Subsequent experiments on D-Asp(OBzl)-Ala [Taub *et al.*, 1997b] showed that it inhibited [^{14}C]-Gly-Sar uptake with an IC_{50} of 2.62±0.35 mM, is rapidly taken up into Caco-2 cells, and is transported in the apical-to-basolateral direction across a Caco-2 monolayer, with both uptake and transport inhibited by Gly-Pro. Asp(OBzl)-Sar had an IC_{50} of 0.014±0.007 mM but was not taken up or transported even though the IC_{50} was significantly lower than that for D-Asp(OBzl)-Ala. It was concluded that the degree of affinity for the dipeptide is not necessarily indicative of the compound's ability to taken up *via* the dipeptide transporter.

Computer-aided conformational analysis of a number of β-lactams and ACE inhibitors was used to identify structural requirements for recognition by the dipeptide transporter [Swaan & Tukker, 1997]. The active analogue approach used required the compounds analysed to be structurally homologous and have a low level of conformational flexibility. Many natural dipeptides contain many freely rotatable bonds and so were excluded from the study.

Compounds that lacked a free α-amino group were transported but compounds that lacked a free α-carboxyl group (methyl or acetyl esters) were not. The diacid ACE inhibitors (*e.g.* enalaprilat) have affinity but are not transported while their monoesters are (*e.g.* enalapril). It was concluded that the presence a β-carboxyl group reduces affinity for the transporter.

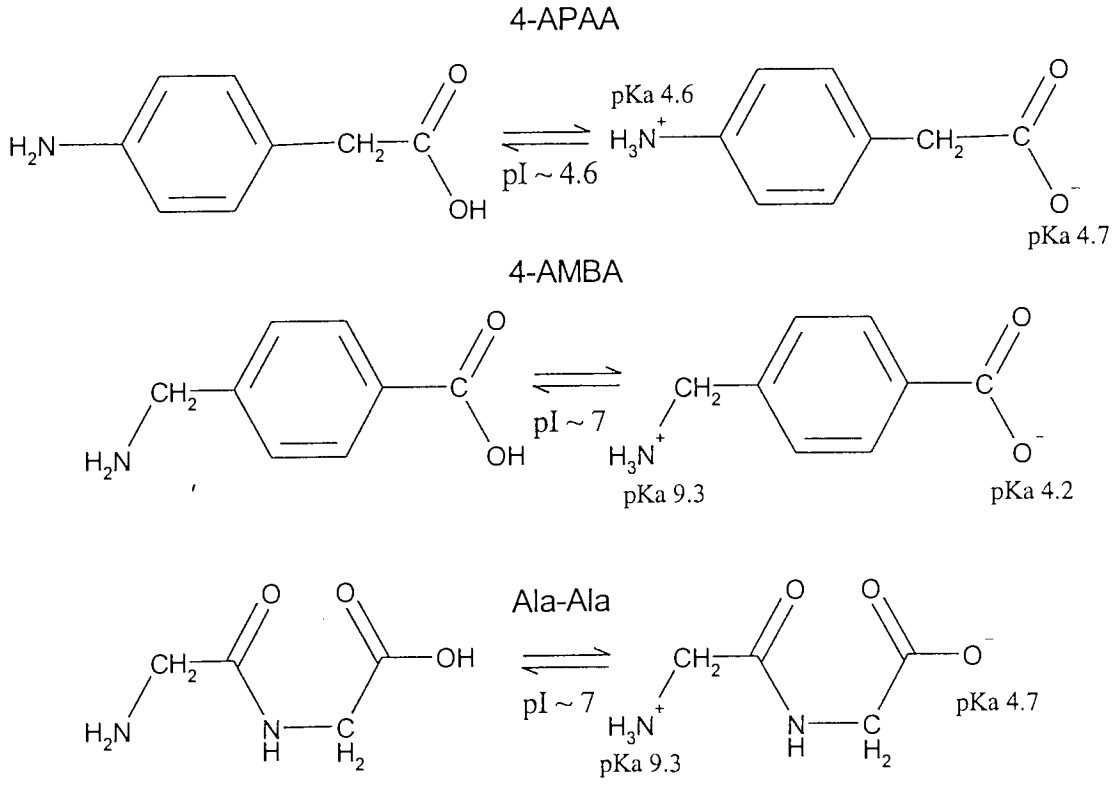
Compounds that are structurally related to substrates of the dipeptide transporter but have no affinity for the transporter themselves, included compounds with positively charged side-chains or hydrogen-acceptor groups close to the α-carboxyl group. It was concluded

that either hydrogen bond formation or charge-charge interactions reduces affinity for the transporter. Compounds needed a peptide bond and a free α-carboxyl group to be transported by the dipeptide transporter but side-chains can reduce or abolish this affinity.

Arphamenine A, an analogue of the dipeptide Arg-Phe with the peptide bond nitrogen replaced by a methylene group to form a ketomethylene group, has been shown to be transported by the dipeptide transporter [Enjoh *et al.*, 1996]. It was concluded that the nitrogen atom in the peptide bond is not necessary for transport.

There has been one study on the necessity of the peptide bond using 4-aminophenylacetic acid (4-APAA) [Temple *et al.*, 1998]. This compound has no peptide bond yet it inhibited the uptake of dipeptides, and was transported in Xenopus oocytes expressing PepT1. However, another study by the same group [Meredith *et al.*, 1998] on 4-aminomethylbenzoic acid (4-AMBA) found that although it inhibited uptake of other dipeptides, it was not transported itself. The structures of both compounds and Ala-Ala are shown in Figure 3.1 below.

Figure 3.1 The structures of 4-aminophenylacetic acid (4-APAA), 4-aminomethylbenzoic acid (4-AMBA) and Ala-Ala



As both 4-APAA and 4-AMBA are structurally similar to Ala-Ala, the fact that they inhibited dipeptide uptake was not unexpected. Therefore a peptide bond may not be necessary to inhibit dipeptide uptake. However 4-AMBA has a pI (pH at which the molecule has no net charge) similar to the pI for Ala-Ala (and the pH of the experiment) yet is not transported, while 4-APAA has a much lower pI but is transported. The results imply that transport does not simply correlate with pI.

There has been one study on the necessity of the peptide bond carbonyl group using the ACE inhibitor enalapril and enamipril (containing a reduced peptide bond) and rat ileum in Ussing chambers [Schoenmakers *et al.*, 1999]. Enalapril was actively transported but enamipril was not although it did inhibit the active transport of amoxycillin. It was concluded that the carbonyl group is necessary for transport but not for affinity for the dipeptide transporter.

A series of ACE inhibitors has been assessed to identify structural preferences for affinity [Moore *et al.*, 2000]. The template used was SQ-29852 and divided into six domains of which four were varied and tested for affinity. Domain D was equivalent to position 4 of the prolyl residue of Gly-Pro. All the variants tested had a higher affinity for the transporter than SQ-29852. Hydroxyproline (replacing the H with an OH) had a non-significant increase in affinity, however the addition of a lipophilic aliphatic group (e.g. cyclohexyl) caused a ten-fold increase in affinity. Replacement with a methyl group had an enhanced affinity but the addition of a benzyl group to the methyl group ($CH_2C_6H_6$) had no extra effect. Domain E was equivalent to the α-carboxyl group in Gly-Pro but only a limited number of variants were tested. The benzyl ester of the carboxyl group (forming a neutrally charged molecule) had an increased affinity compared to the parent compound (with a negative molecular charge) SQ-29852 (0.05±0.01 mM vs. 0.68±0.17 mM). This is in contrast to the results stated previously [Hidalgo *et al*, 1995; Swaan & Tukker, 1997] that the presence of a free carboxyl group is necessary although only the effect of methyl esters had previously been tested.

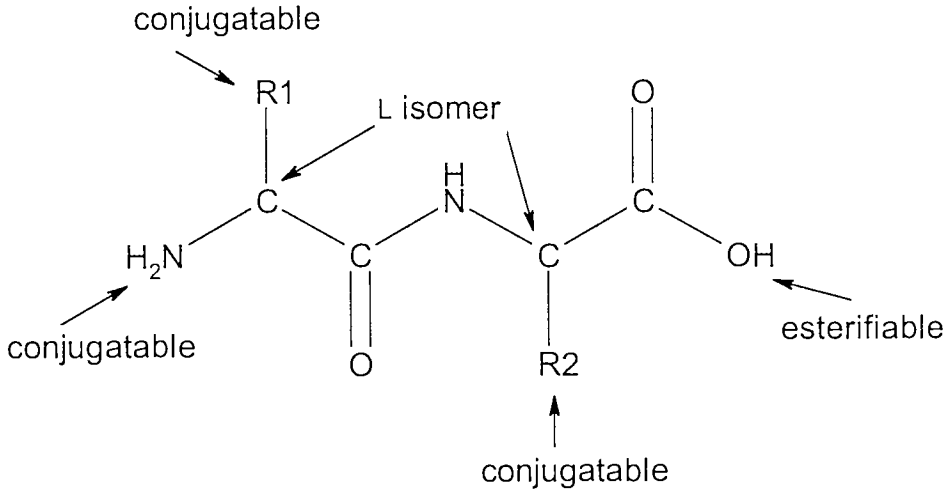
3.1.3 Current understanding of the structural requirements for in vitro *uptake via the dipeptide transport system*

The previous studies can be summarised as follows:

- Affinity for the dipeptide transporter does not correspond to transport by the dipeptide transporter.
- The lack of either the peptide bond carbon or nitrogen results in inhibition but not transportation.
- The α-amino group is not needed for inhibition, and the conjugation of fatty acids to the α-amino group can increase inhibition.
- Generally modifying the α-carboxyl group resulted in a decrease in affinity except for benzyl esters. Reducing it to an alcohol or creating a methyl or ethyl ester reduced affinity. The creation of the benzyl ester of Gly-Gly caused the smallest reduction in affinity of all the esters examined in one study and the creation of the benzyl ester of a negatively charged ACE inhibitor caused a significant increase in affinity in another.
- The LL-isomer has the greatest uptake via the dipeptide transporter followed by the DL- and LD-isomers. The DD-isomer is not transported.
- Charged side-chains have produced contradictory results. One study concluded that hydrogen-accepting side-chains or with a charge of +1 have no affinity, while another concluded that neutral or +1 charged molecules have a higher affinity for the dipeptide transporter than molecules with a charge of –1 or +2.
- The benzyl esters of side-chains are better inhibitors than the parent dipeptide containing the free carboxyl group. ACE inhibitors containing two free acid groups are not transported but those containing β-carboxyl esters (i.e. one free acid group) are transported.
- The presence of one sarcosyl residue reduces affinity but the presence of two abolishes it.
- Modifying the prolyl ring increasing affinity. The addition of a cyclohexyl ring created the best inhibitor, but the addition of a benzyl group ($CH_2C_6H_6$) had exactly the same increase in affinity as the addition of a methyl group (CH_3).

These structural requirements are shown in Figure 3.2 below.

Figure 3.2 Summary of optimal structural requirements for uptake *via* the dipeptide transporter



3.1.4 Areas for investigation

Although there is a huge number of possible derivatives that could be examined, three main areas will be studied:

1. The effect on affinity of uncharged dipeptide side-chains. This will include the effect of the length of the side-chain as well as the presence of sulphur and oxygen atoms in the side chain. This will identify the optimum structure for uncharged inhibitors.
2. The effect of charged side-chains on affinity to identify the optimum charge to have on charged inhibitors.
3. The effect of modifying the α-carboxyl group. This will identify changes that can be made to increase affinity while reducing the charge on the molecule.

3.1.5 Aim and objectives of chapter

The aim of this chapter is to identify structure-activity relationships for the intestinal dipeptide transporter by developing a rapid screening system and using it to determine the affinities of a number of dipeptide derivatives. The template for the dipeptide derivatives will be L-X-L-Pro (where X is a naturally occurring amino acid residue) in order to maximise inhibition, as it contains two L-isomers joined by a peptide bond.

The objectives for this chapter are:

- To identify the optimum conditions for the inhibition of dipeptide uptake into Caco-2 cells
- To identify the effect on affinity of various esters of the α-carboxyl group
- To identify the effect on affinity of uncharged dipeptide side-chains
- To identify the effect on affinity of charged dipeptide side-chains and their esters
- To identify the effect on affinity of dicarboxylic acids joined to a prolyl residue by a peptide bond and sarcosyl containing compounds (non-dipeptide derivatives), to study the necessity of an α-amino group
- To identify the effect on affinity of modifying the prolyl residue in dipeptides to create benzyl ethers
- To identify molecular parameters (derived from molecular modelling) that correlate to IC_{50}

3.2 Materials and Methods

General materials and methods can be found in Chapter 2.

3.2.1 Source of dipeptide derivatives

The dipeptide derivatives tested in this chapter were synthesised by the Medicinal Chemistry Research Group at Aston University.

3.2.2 Effect of DMSO on Gly-L-Pro uptake

The basic protocol was as described in section 2.2.3.2. The Gly-L-Pro solution contained a range of concentrations of DMSO (0-5%).

3.2.3 Standard uptake protocol

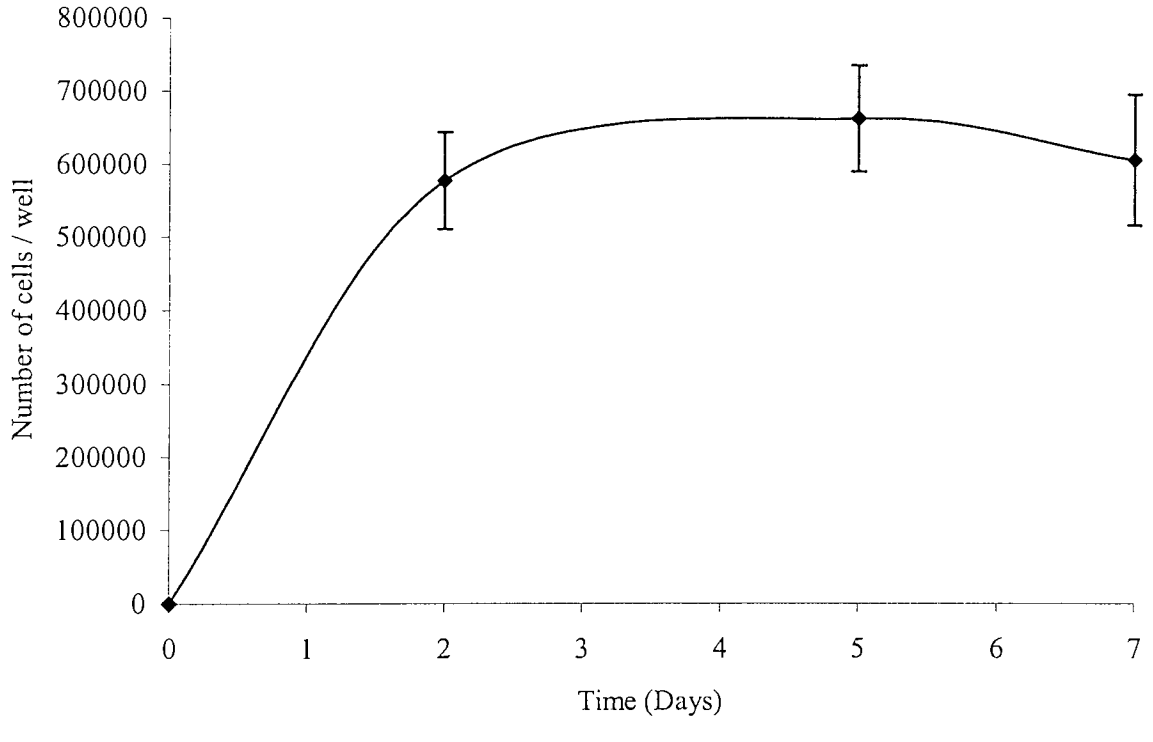
The basic protocol was as described in section 2.2.3.2. The Gly-L-Pro solution additionally contained 1% DMSO.

3.3 Results and discussion

3.3.1 Cell development

In order to prove that cells reach confluence in one week, the number of cells in a well was determined over a week. Figure 3.3 (below) shows that the cell number reaches a plateau and is constant by seven days post-seeding in a 24-well plate. A BCA protein assay, performed on cells seven days post-seeding in a 24-well plate, found 226(±40) μg protein $well^{-1}$. This number was used to allow results to be expressed as mg $protein^{-1}$, where appropriate.

Figure 3.3 Effect of time on Caco-2 cell number in each well



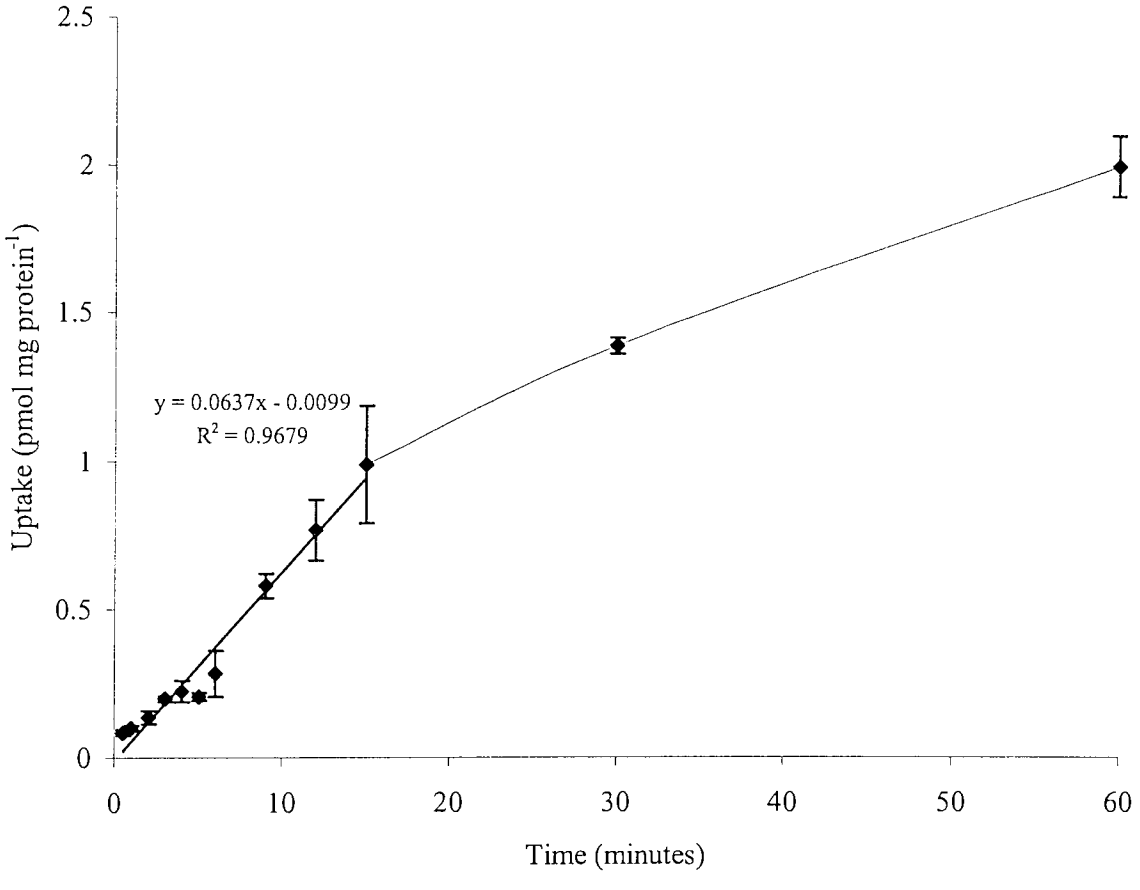
Data are mean and standard deviation of results from four wells

3.3.2 Optimum conditions for uptake of Gly-L-Pro

3.3.2.1 The uptake of Gly-L-Pro against time

The kinetic profile of Gly-[^{3}H]L-Pro uptake is shown in Figure 3.4 below. The results show an initial linear phase (0 – 15 minutes) followed by a slower non-linear phase (15 – 60 minutes). This may be due to a decreased concentration gradient across the membrane over time. For future experiments, an incubation period of three minutes was selected for the competition studies as that duration falls within the initial linear uptake phase.

Figure 3.4 Kinetics of Gly-[^{3}H]L-Pro uptake into Caco-2 cells

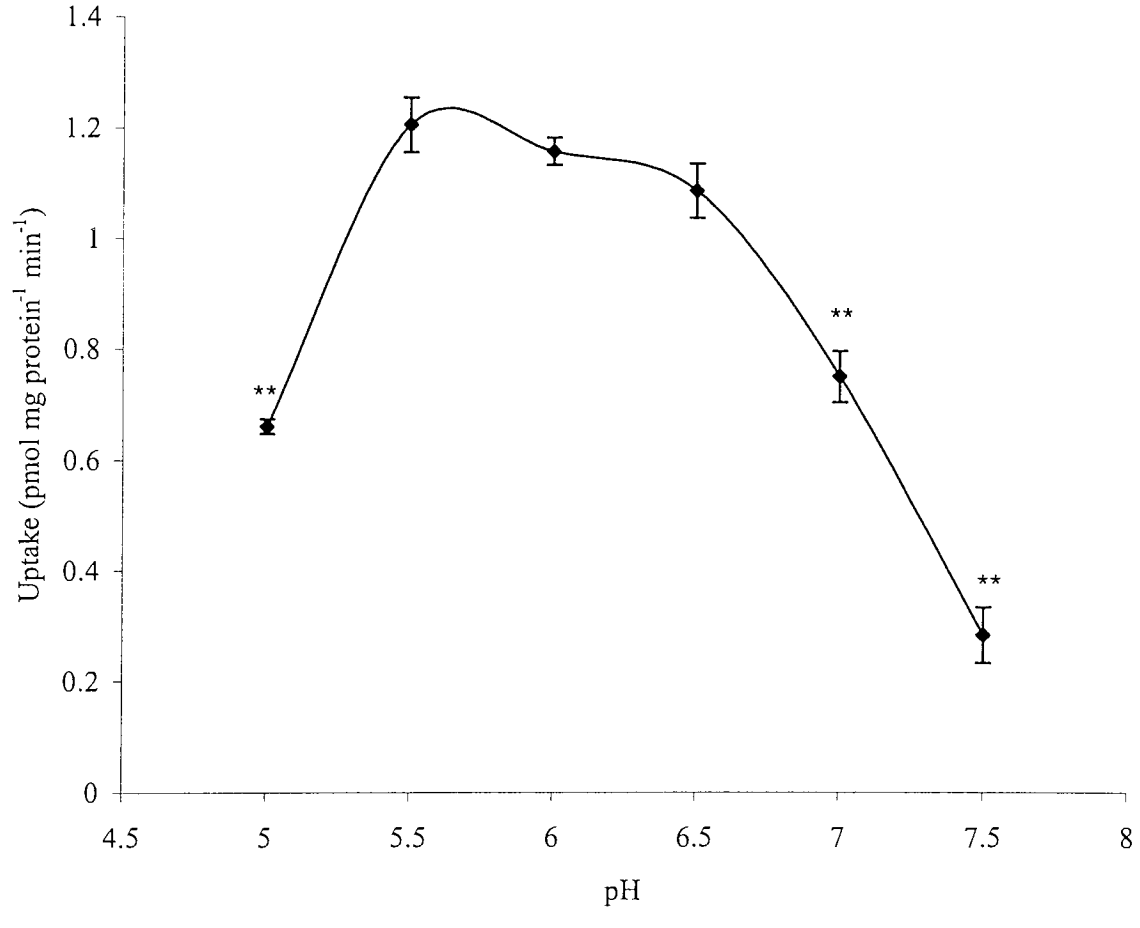


Data are mean and standard deviation of results from four wells

3.3.2.2 The uptake of Gly-L-Pro over a range of pH values

The uptake of Gly-L-Pro in the system tested showed clear pH-dependence (Figure 3.5 below). The maximum uptake was at pH 5.5. A previous report [Thwaites *et al.*, 1993] had found optimum uptake at pH 6. This was due to the dipeptide transporter being driven by a proton gradient, with the optimum pH being pH 6. However an ANOVA analysis of the data presented in Figure 3.5 found no significant difference in the uptake at pH 5.5, 6 or 6.5 therefore the optimum pH for uptake may agree with the previous study. For future experiments, a pH6 transport media will be used.

Figure 3.5 Effect of pH on Gly-[^{3}H]L-Pro uptake

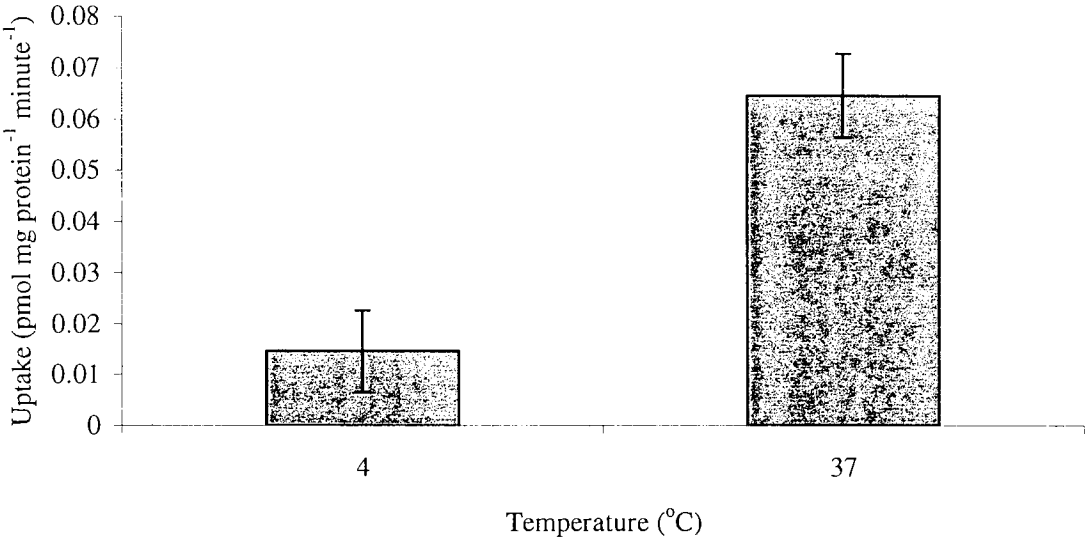


Data are mean and standard deviation of results from four wells.
** Denotes a very significant ($p < 0.01$) reduction in Gly-Pro uptake compared to uptake at pH 6

3.3.2.3 The effect of different temperatures on the uptake of Gly-L-Pro

Active transport processes are inhibited by a reduction in temperature. The uptake of Gly-L-Pro was measured at 4°C and at 37°C. The results are shown in Figure 3.6 below showing a lower uptake at 4°C than at 37°C. A two-tailed Student's t-test had a P value of 0.0003, which is considered extremely significant. Therefore uptake was significantly reduced at 4°C confirming that that Gly-L-Pro uptake is an active transport process.

Figure 3.6 Effect of temperature on Gly-[^{3}H]L-Pro uptake



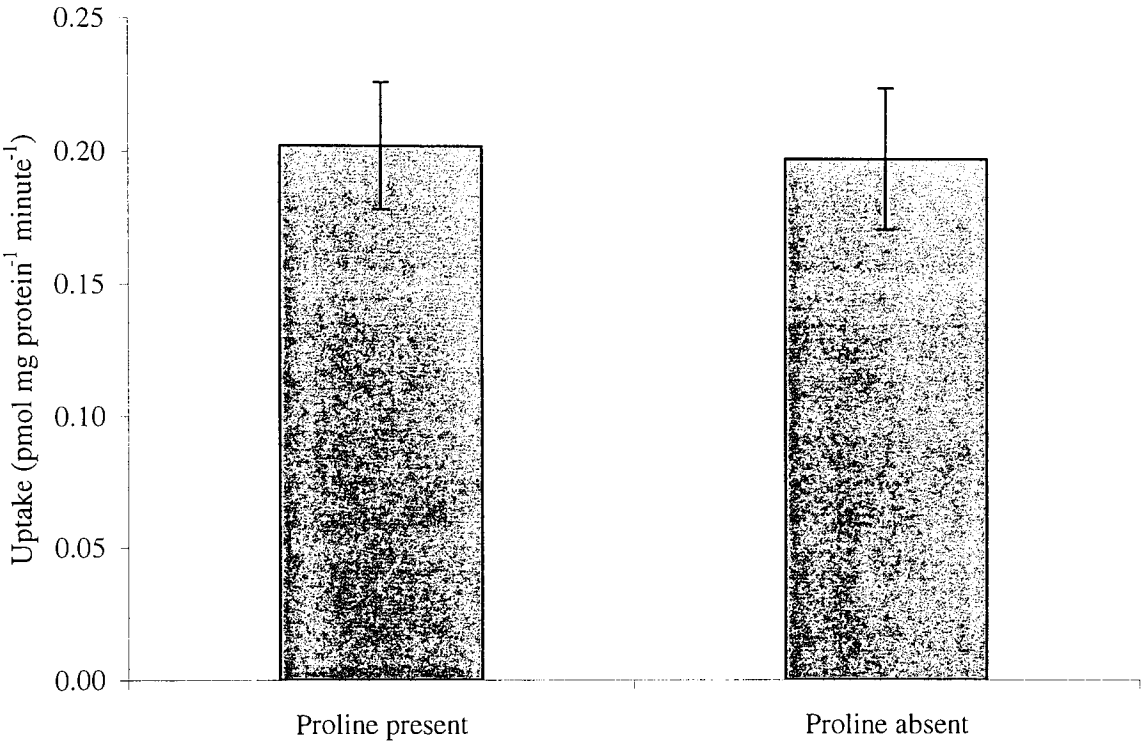
Data are mean and standard deviation of results from four wells.

3.3.2.4 The effect of proline on the uptake of Gly-L-Pro

Caco-2 cells have been reported to produce prolidase, an enzyme that is responsible for X-Pro hydrolysis (where X = amino acid) [Hu *et al.*, 1994]. Prolidase is primarily an enzyme of the cytosolic domain that has been demonstrated to hydrolyse the dipeptide L-Phe-L-Pro. Gly-[^{3}H]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.*, 1992] that 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. Excess L-Pro has also been shown to inhibit prolidase activity by 66 % [Hu *et al.*, 1994] and therefore acts as a preventative measure to hydrolysis.

The uptake of Gly-[^{3}H]L-Pro in the presence or absence of 10mM L-Pro was calculated and the results are shown in Figure 3.7 below. The results do not show a significant difference in the presence or absence of 10mM L-Pro (a Student's t-test had a p-value of 0.8078 considered not significant). However 10 mM L-Pro was included in the incubation solution for all future experiments as a precaution.

Figure 3.7 Effect of proline on Gly-[^{3}H]L-Pro uptake

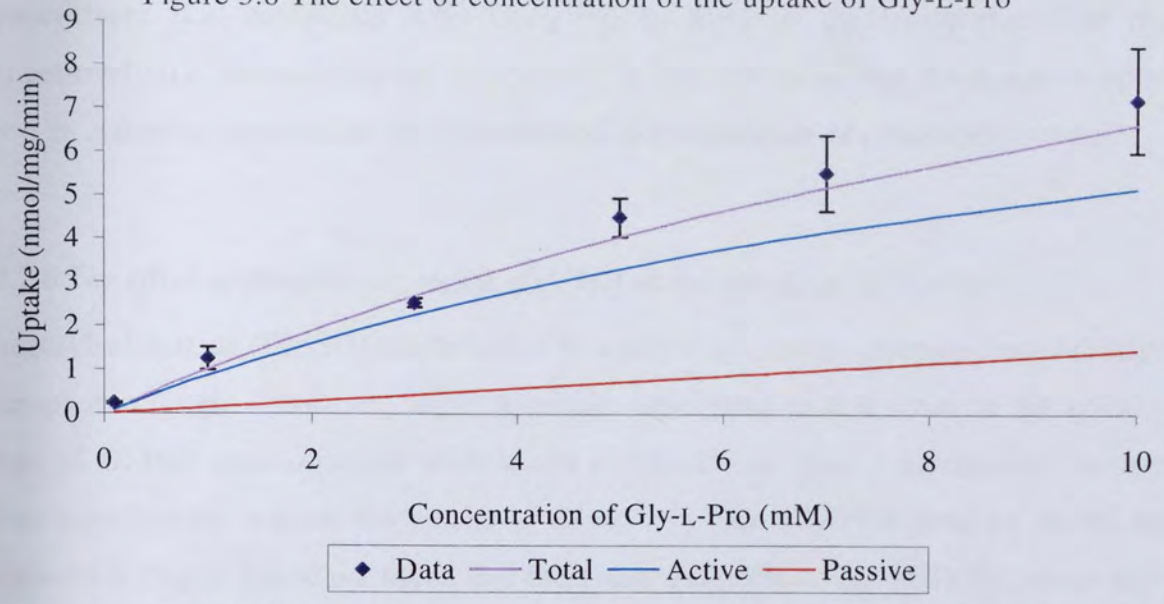


Data are mean and standard deviation of results from four wells.

3.3.2.5 The effect of concentration on the uptake of Gly-L-Pro

The uptake of Gly-L-Pro was calculated over a range of concentrations. The uptake of Gly-L-Pro has been shown to involve active and passive uptake processes [Moore *et al.*, 2000]. The adapted Michaelis-Menton equation (Equation A1.2) was therefore used to calculate kinetic parameters. Using Fig P software, V_{max} was calculated to be 11.39 nmol/mg/min, K_m 12.1 mM and K_d 0.147 nmol/mg/min/mM. The results are shown in Figure 3.8 below. The next study to investigate the effect of the presence of an inhibitor (1mM SQ29852) on the uptake of Gly-L-Pro by repeating the previous study (the effect of concentration on uptake) with the inhibitor present in all solutions.

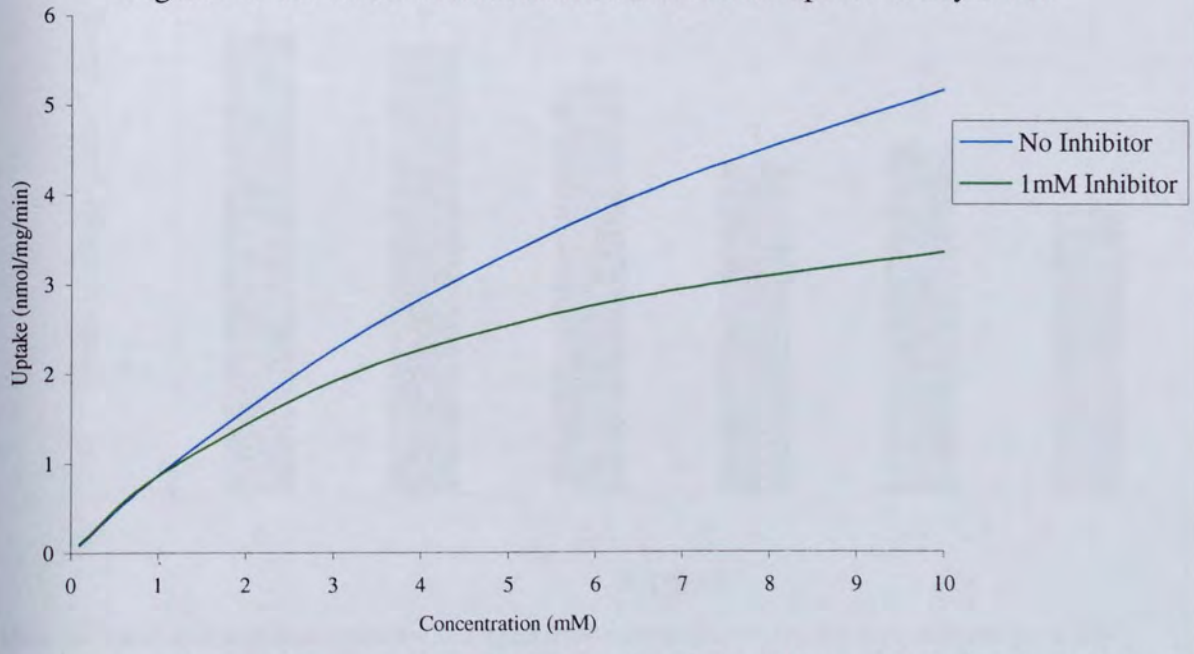
Figure 3.8 The effect of concentration of the uptake of Gly-L-Pro



Data are mean and standard deviation of results from four wells. Total, Active and Passive are the predicted values based upon the calculated Vmax, Km and Kd values

It was assumed that the amount of passive uptake was identical in both studies (*i.e.* K_d was 0.147 nmol/mg/min/mM in both studies). Fig P software was then used to determine the other parameters. It calculated a new V_{max} of 4.91 nmol/mg/min and new K_m of 4.69 mM. The parameters were then used to calculate the level of active uptake. Figure 3.9 below shows the calculated active uptake in the presence and absence of the inhibitor.

Figure 3.9 The effect of an inhibitor on the active uptake of Gly-L-Pro



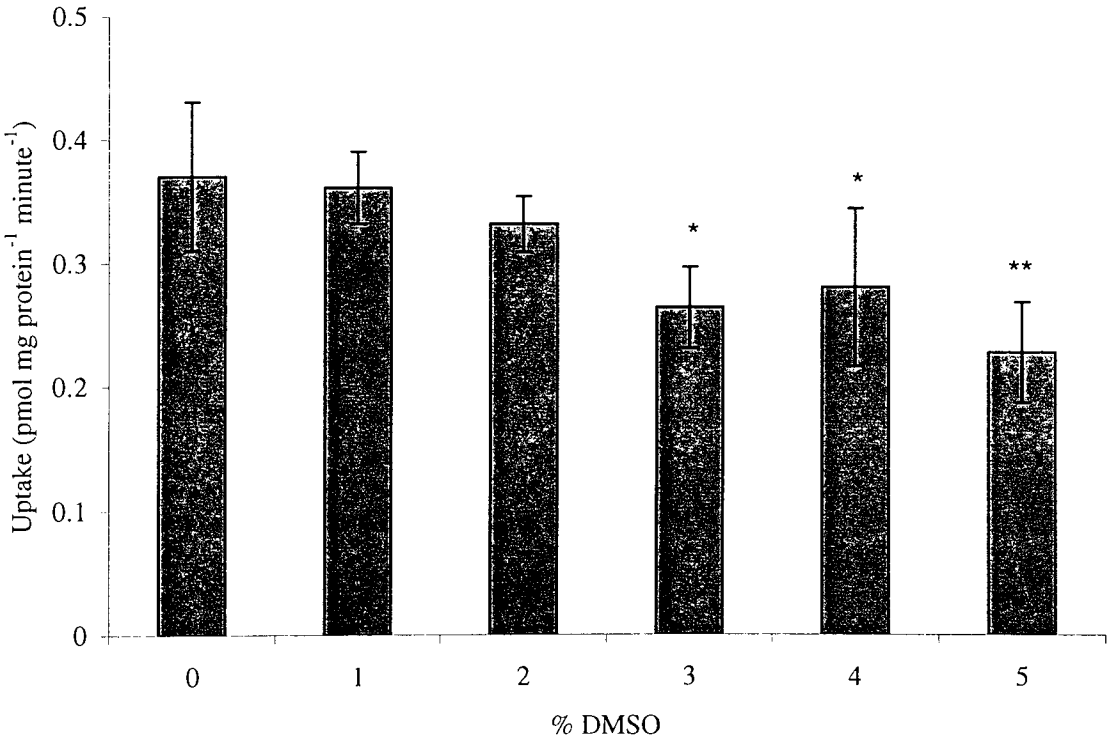
Active uptake values are the predicted values based upon the calculated Vmax and Km values from calculated uptake in the presence and absence of 1mM SQ29852 in uptake media.

The reduction in active uptake in the presence of the inhibitor implies that the inhibitor is effective. The reduction in both V_{max} and K_m implies that the inhibitor is inhibiting competitively (*i.e.* competing with Gly-L-Pro to bind to the transporter) and non-competitively (*i.e.* deactivating the transporter). It was concluded that the dipeptide uptake is *via* an active transporter that may be inhibited in the presence of other compounds.

3.3.2.6 The effect of dimethylsulphoxide (DMSO) on the uptake of Gly-L-Pro

Dimethylsulphoxide (DMSO) can be added to aqueous systems to increase the solubility of hydrophobic drugs. However, above a certain concentration it is toxic to the cells. A range of DMSO concentrations were tested to identify at what concentration the toxic effect significantly reduces the uptake of Gly-L-Pro. An ANOVA analysis of the data presented in Figure 3.6 below found that there was a significant ($p<0.05$) difference at 3% and above. Therefore transport media (pH6) containing 1% DMSO was used in all future experiments to increase the solubility of competitor compounds. The size of the increase in the solubility of Gly-L-Pro in 1% DMSO compared to 0% DMSO is unknown.

Figure 3.10 Effect of DMSO on Gly-[^{3}H]L-Pro uptake

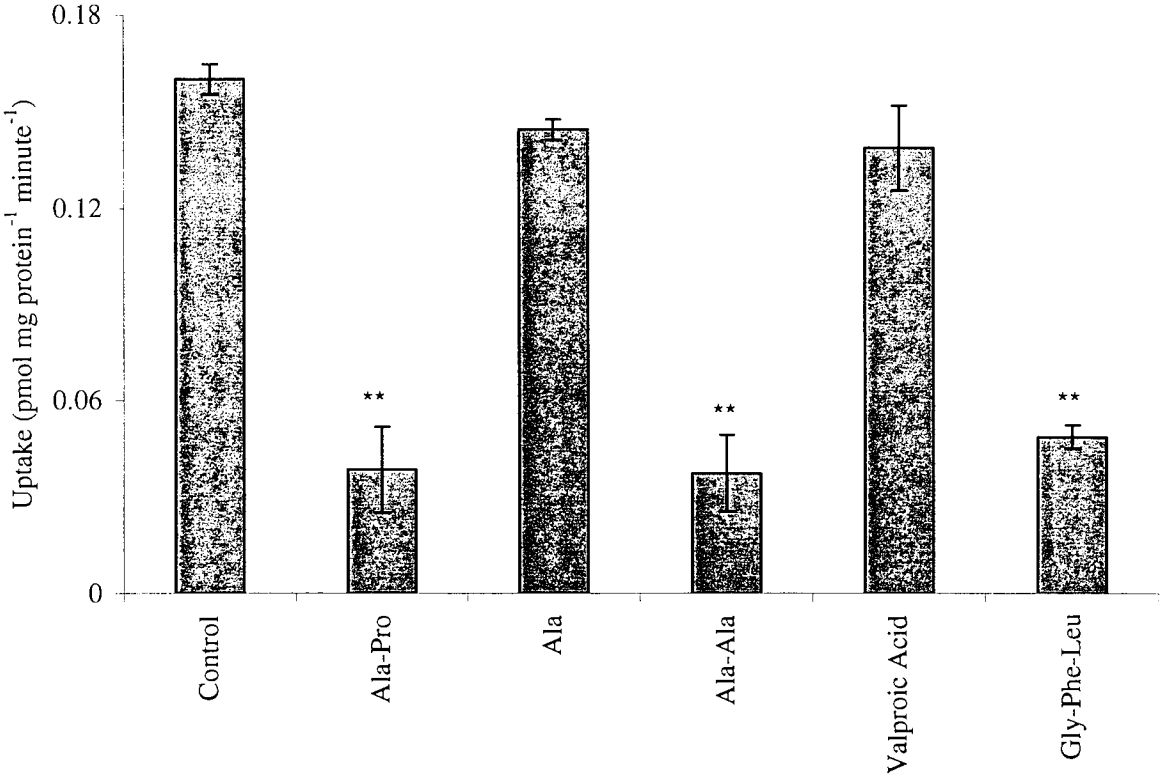


Data are mean and standard deviation of results from four wells. * Denotes a significant ($p< 0.05$) reduction from Gly-Pro uptake at 0% DMSO. ** Denotes a significant ($p< 0.01$) reduction from Gly-Pro uptake at 0% DMSO

3.3.2.7 The effect of a range of potential inhibitors on the uptake of Gly-L-Pro

A number of transporters have been identified in Caco-2 cells. Potentially Gly-[^{3}H]L-Pro could be hydrolysed to [^{3}H]L-Pro and the radioactive label be transported through the amino acid transporter. The Gly-[^{3}H]L-Pro could also potentially be transported through the monocarboxylic acid transporter as it has a free carboxylate group. A number of potential inhibitors of Gly-[^{3}H]L-Pro uptake were tested and the results shown in Figure 3.11 below.

Figure 3.11 Effect of a range of compounds on Gly-[^{3}H]L-Pro uptake



Data are mean and standard deviation of results from four wells.
** Denotes a very significant ($p < 0.01$) reduction from control Gly-Pro uptake

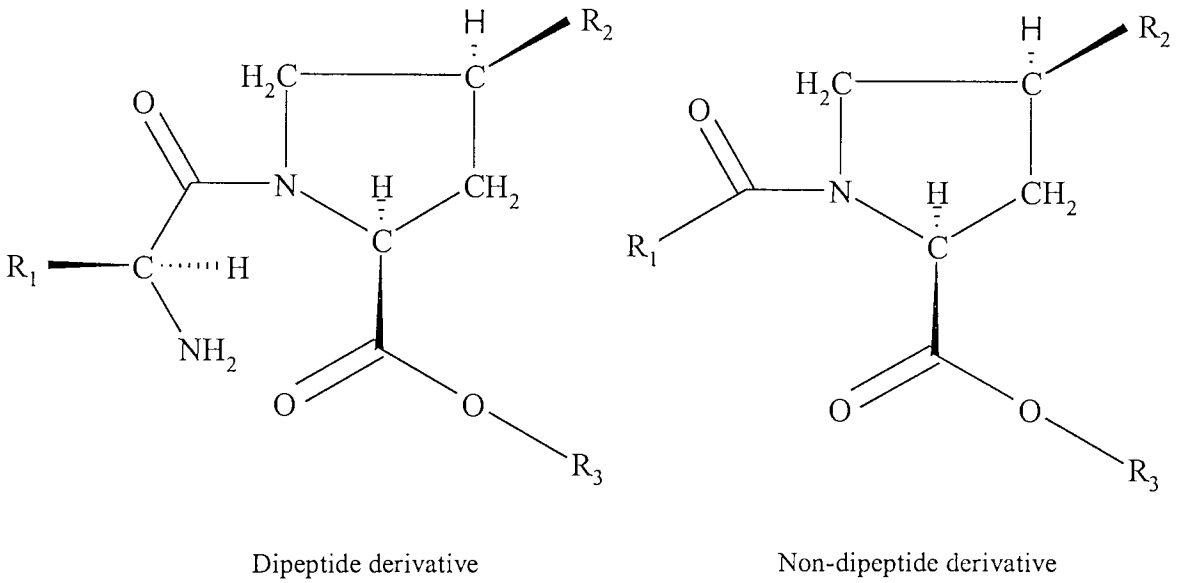
An ANOVA analysis of the data presented in Figure 3.11 found that two dipeptides and one tripeptide (Ala-Pro and Ala-Ala) significantly ($p<0.01$) inhibited uptake. Alanine inhibits the amino acid transporter and valproic acid inhibits the monocarboxylic transporter yet neither had a significant ($p>0.05$) effect on uptake. It was concluded that Gly-[^{3}H]L-Pro is transported by the dipeptide transporter but not the amino acid transporter or the monocarboxylic acid transporter.

3.3.3 The effect of dipeptide derivatives on Gly-L-Pro uptake

3.3.3.1 The structures of the derivatives

The chemical structure of the template used to make dipeptide derivatives was based on Gly-L-Pro and is shown in Figure 3.12 below. The chemical structures were varied at three different positions (R_1, R_2 and R_3).

Figure 3.12 The template structures of the dipeptide and non-dipeptide derivatives



Dipeptide derivative

Non-dipeptide derivative

The chemical structure of the template used to make non-dipeptide derivatives is similar to the template for dipeptide derivatives. The template is still based on X-Pro but in this case X is a carboxylic acid not an amino acid.

Changing groups at the R_1 position resulted in changing the side-chain of the dipeptide X-L-Pro where X is the amino acid being changed. The different side-chains tested are shown in Table 3.1 below. Changing groups at the R_2 position resulted in changing the five-membered ring in the proline residue. The two different groups tested are shown in Table 3.2 below. Changing groups at the R_3 position resulted in esterifying the carboxylate group on the proline residue. The different esters tested are shown in Table 3.3 below.

Table 3.1 Structures of different R_1 groups tested

(See Figure 3.12 for structures of molecules)

In dipeptide derivatives:

R_1 Structure	Residue Name
H	Glycyl
CH_3	Alanyl
CH_2CH_3	Abutyl
$CH_2CH_2CH_3$	Norvalyl
$CH_2CH_2CH_2CH_3$	Norleucyl
CH_2SH	Cysteinyl
CH_2SCH_3	Methionyl

R_1 Structure	Residue Name
CH_2OH	Seryl
$CH_2CH_2CH_2CH_2NH_2$	Lysyl
CH_2COOH	Aspartyl
$CH_2COOC_6H_6$	Aspartyl (O-Benzyl)
CH_2CH_2COOH	Glutamyl
$CH_2CH_2COOC_6H_6$	Glutamyl (O-Benzyl)

In non-dipeptide derivatives:

R_1 Structure	Residue Name
CH_2NHCH_3	Sarcosyl
$C_3H_6COOC_2H_5$	Suboic(O-Ethyl)
$C_5H_{10}COOCH_3$	Adipic(O-Methyl)
$C_6H_{12}COOC_2H_5$	Pimoic(O-Ethyl)

Table 3.2 Structures of different R_2 groups tested (for both sets of derivatives)

(See Figure 3.12 for structures of molecules)

R_2 Structure	Residue Name
H	Proline
$OCH_2C_6H_6$	Benzyloxyproline

Table 3.3 Structures of different R_3 groups tested (for both sets of derivatives)

(See Figure 3.12 for structures of molecules)

R_3 Structure	Residue Name
H	Carboxylic acid
CH_3	Methyl ester
$CH_2C_6H_6$	Benzyl ester
$CH(C_6H_6)_2$	Diphenylmethyl ester

3.3.3.2 Structure-activity relationships

The effect of the presence of sixty-four dipeptide and non-dipeptide derivatives on the uptake of Gly-L-Pro was investigated. An IC_{50} value for each compound was then calculated. A number of molecular parameters for each compound were also derived (using the CAChe computer programme). This section examines the relationship between the chemical structure of the derivatives and their IC_{50} values. The next section (Section 3.3.3.3) examines the relationship between the molecular parameters and their IC_{50} values.

The results can be grouped into series of related compounds to identify patterns. The groups that will be examined are detailed below:

- The sequential addition of a methyl group to the R_1 position to create a series of dipeptides
- The methyl and benzyl esterification of the R_3 carboxylic acid of the dipeptides
- Dipeptides containing a sulphur atom in the R_1 side-chain
- Dipeptides containing an oxygen atom in the R_1 side-chain
- Dipeptides containing nitrogen atoms in the R_1 side-chain
- Dipeptides containing a carboxyl group in the R_1 side-chain
- Dipeptides containing charged atoms
- Non-dipeptide derivatives
- Derivatives containing diphenylmethyl esters
- Dipeptides containing a benzyl ester at the R_2 position

3.3.3.2.1 The effect of the sequential addition of a methyl group to the R_1 position

Changing groups at the R_1 position resulted in changing the side-chain of the dipeptide X-L-Pro. When R_2 and R_3 were both H (*i.e.* the parent dipeptide) increasing the chain length of the side chain tended to increase the IC_{50}, however the norleucyl residue had the lowest IC_{50} value in the series. These results are shown in Table 3.4 below.

An ANOVA analysis of the results presented in Table 3.4 had a P value of <0.0001, considered extremely significant. The variation seen is significantly greater than expected by chance.

Table 3.4 The effect of increasing chain-length on the IC_{50} value

(See Figure 3.12 for structure of molecule)

R_1 Structure	Side-chain name	IC50 (±SD) mM	cLogP
H	Glycyl	0.458 (0.079)	-1.38
CH_3	Alanyl	0.487 (0.042)	-0.84
CH_2CH_3	Abutyl	0.779 (0.180)	-0.37
$CH_2CH_2CH_3$	Norvalyl	1.208 (0.136)	0.02
$CH_2CH_2CH_2CH_3$	Norleucyl	0.299 (0.027)	0.42

Dipeptides where R_2=H and R_3=H. Data are mean and standard deviation of results from four wells. cLogP calculated using CAChe.

A Tukey-Kramer Multiple Comparisons Test found that there was no significant difference between the IC_{50}s of glycyl and alanyl, a significant difference between alanyl and abutyl ($p<0.05$), and a very significant difference between glycyl and abutyl ($p<0.01$). The norvalyl IC_{50} was significantly greater than that for glycyl, alanyl, abutyl and norleucyl (all $p<0.001$). These IC_{50} results can be summarised as glycyl < alanyl < abutyl < norvalyl >> norleucyl. Therefore it can be concluded that increasing the chain length increases the hydrophobicity of the molecule and therefore reducing its ability to inhibit uptake of Gly-[^{3}H]L-Pro, however a side chain of four carbons (norleucyl) increased inhibition. This could be due to the norleucyl chain being long enough to reach a binding site on the transporter or being hydrophobic enough (cLogP >0) to bind to one. A study [Tateoka *et al.*, 2001] *to* determine the significance of hydrophobicity for recognition by PepT1, synthesized dipeptide analogues conjugating the E-amino group of Lys in Val-Lys with aliphatic carboxylic acids: acetic acid (C2), propanoic acid (C3), pentanoic acid (C5), hexanoic acid (C6), and decanoic acid (C10). The affinities of these conjugates were estimated by their inhibition of the accumulation rate of Gly-Sar. With the increase in length of the hydrocarbon chain of the conjugates, i.e., in the hydrophobicity of the conjugates, the inhibition strengthened. Dixon-Webb plot analysis of the inhibition by the C10-conjugated dipeptide showed competitive inhibition. The results of the study confirmed that the hydrophobicity of substrates/inhibitor is one of the factors in the recognition by PepT1. The results presented in Table 3.4 show that increasing the hydrophobicity of the N terminal residue (the Gly of Gly-Pro) does not result in the simple increase in affininy that increasing the hydrophobicity of the C-terminal residue (the Lys of Val-Lys).

Key Finding 1: Increasing the side-chain length of simple dipeptides at the R_1 position increased IC_{50} (reducing affinity) until the chain was four-carbons long, where the IC_{50} decreased (increasing affinity).

3.3.3.2.2 The effect of the methyl and benzyl esterification of the R_3 carboxylic acid

The results can be grouped to show the effect of changing the R_3 position *i.e.* creating methyl and benzyl esters from a carboxylic acid. The results for five different dipeptides are shown in Table 3.5 below.

Table 3.5 The effect of esterification of the R_3 group on the IC_{50} value

R_1 Structure	Side-chain name	IC50 (±SD) mM		
		$R_3 = H$	$R_3 = CH_3$	$R_3 = CH_2C_6H_6$
H	Glycyl	0.458 (0.079)	1.575 (0.137)	0.627 (0.355)
CH_3	Alanyl	0.487 (0.042)	1.798 (1.030)	1.829 (0.320)
CH_2CH_3	Abutyl	0.779 (0.180)	5.268 (2.679)	0.438 (0.132)
$CH_2CH_2CH_3$	Norvalyl	1.208 (0.136)	1.161 (0.131)	0.686 (0.054)
$CH_2CH_2CH_2CH_3$	Norleucyl	0.299 (0.027)	0.786 (0.071)	0.438 (0.044)

Dipeptides where R_2=H. Data are mean and standard deviation of results from four wells.

The effect of varying the R_1 group on the methyl esters has a similar pattern to the effect of varying the R_1 group on the carboxylic acids (key finding 1 above). However the IC_{50} peaked with the abutyl residue and then decreased. Norleucyl still had the lowest IC_{50} (glycyl=alanyl<abutyl>norvalyl=norleucyl) although it was only significantly ($p<0.01$) smaller than the abutyl. There was a similar pattern in the benzyl esters, although the IC_{50} peaked with alanyl (significantly ($p<0.001$) larger than the other results) and there was no significant ($p>0.05$) difference between the abutyl, norvalyl and norleucyl residues (glycyl<alanyl>abutyl=norvalyl=norleucyl). The IC_{50} value was largest with a three-carbon R_1 group and carboxylic acid, a two-carbon R_1 group and methyl ester, and a one-carbon R_1 group and benzyl ester. This may be due to a substrate of a certain molecular width poorly binding to the transporter. Key Finding 1 was modified to Key Finding 1a.

Key Finding 1a: Increasing the side-chain length of dipeptides at the R_1 position increased IC_{50} until the chain reached a certain length (dependent on an unknown factor), at which point the IC_{50} decreased.

A series of ANOVA analyses were performed on the data presented in Table 3.5. The data on each row were analysed to determine if there was any significant difference between the IC_{50} values for each carboxylic acid, methyl ester and benzyl ester. In each case the ANOVA had a p value <0.05 implying that the results were more varied than expected by chance. These analyses mean that significantly different results were calculated for the esters. This is an indirect method to prove that the esters are stable. If they were not stable then in each case the esters would degrade into the carboxylic acids and identical effects would be seen. Experiments to determine the exact stability of each ester were not performed but should be in the future.

Key Finding 2: Methyl and benzyl esters had significantly different results to the parent carboxylic acids implying that they are stable.

A third key finding was also identified from the pattern of the results presented in Table 3.5.

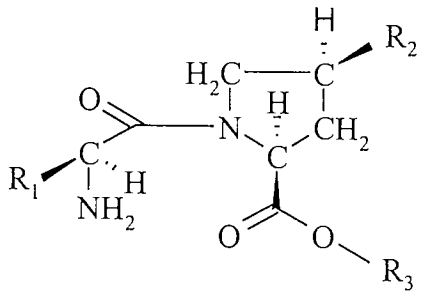
Key Finding 3: The R_3 benzyl esters are better than or equal to the parent acids as inhibitors and that the R_3 methyl esters are the worst inhibitors.

A possible conclusion from this finding was that the affinity of the carboxylic acids is due to an ionic interaction, the affinity of the benzyl esters is due to a hydrophobic interaction but the lack of affinity of the methyl esters is due to the fact that they are neither ionic nor sufficiently hydrophobic.

Table 3.6 below shows the IC_{50}s, calculated logP (cLogP; as a measure of hydrophobicity) and molecular surface area (as a measure of the size of the molecule) for dipeptide derivatives based on increasing the chain length of the R_1 group by the sequential addition of methyl groups. The table also shows the results of varying the R_3 group (carboxylic acid, methyl ester, benzyl ester). The data presented in Table 3.4 previously led to the finding that an R_1 chain-length of more than four carbons had a low IC_{50} value. Nle-Pro has a cLogP >0 and therefore a hydrophobic interaction might have been occurring to

reduce IC_{50}. Figure 3.13 uses the IC_{50} data from Table 3.6 (below) to examine the relationship between IC_{50} and cLogP.

Table 3.6 Effect of increasing the R_1 chain length on dipeptide derivatives*

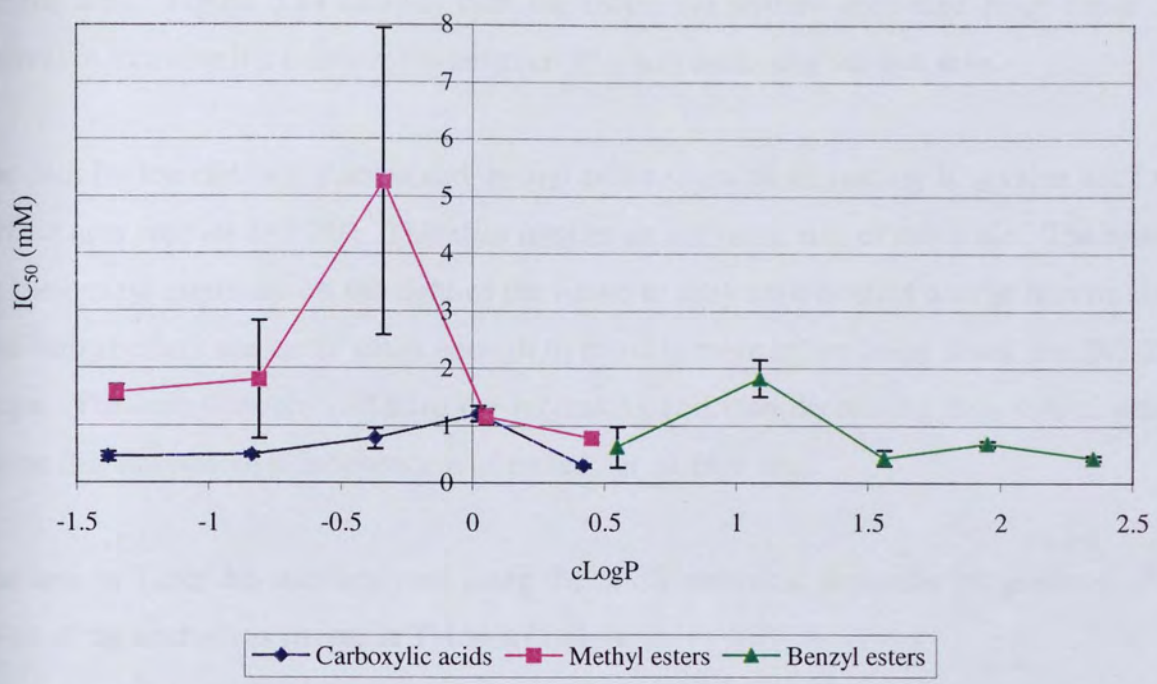


When R_2 = H

Side chain	R_3 = H	R_3 = Me	R_3 = Bzl
R_1 =	Mean IC_{50} (±SD) mM *cLogP* Molecular surface area	Mean IC_{50} (±SD) mM *cLogP* Molecular surface area	Mean IC_{50} (±SD) mM *cLogP* Molecular surface area
Glycyl (H)	0.458 (0.079) *-1.38* 185.79	1.575 (0.137) *-1.35* 206.27	0.627 (0.355) *0.55* 293.84
Alanyl (CH_3)	0.487 (0.042) *-0.84* 195.42	1.798 (1.030) *-0.81* 213.58	1.829 (0.320) *1.09* 298.07
Abutyl (CH_2CH_3)	0.779 (0.180) *-0.37* 205.02	5.268 (2.679) *-0.34* 233.51	0.438 (0.132) *1.56* 319.12
Norvalyl ($CH_2CH_2CH_3$)	1.208 (0.136) *0.02* 225.56	1.161 (0.131) *0.05* 251.52	0.686 (0.054) *1.95* 341.84
Norleucyl ($CH_2CH_2CH_2CH_3$)	0.299 (0.027) *0.42* 246.21	0.786 (0.071) *0.45* 266.89	0.438 (0.044) *2.35* 358.75

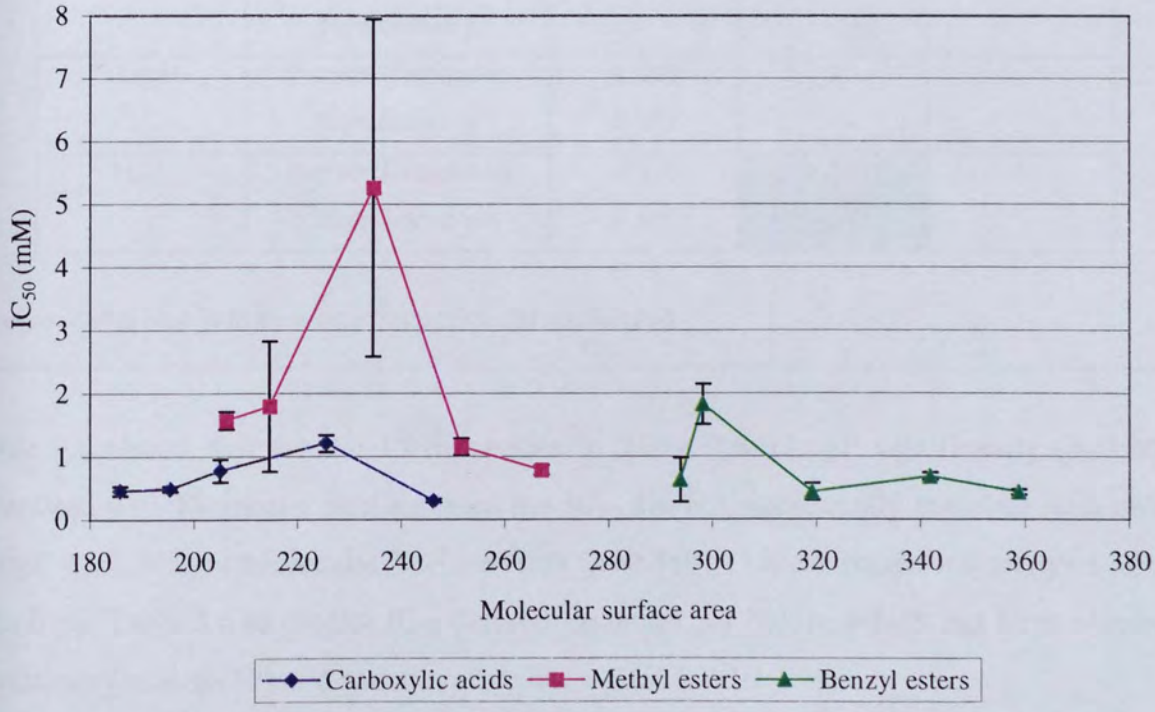
The methyl esters with cLogP >0 (Nva-Pro OMe and Nle-Pro OMe) did have lower IC_{50} values but were only significantly ($p<0.01$) lower than the Abu-Pro methyl ester. All the benzyl esters had IC_{50} values >0 due to the presence of the hydrophobic benzene group. The pattern of increasing then decreasing IC_{50} values is seen in the benzyl esters and therefore is probably unrelated to cLogP.

Figure 3.13 The effect of cLogP on IC_{50} for simple dipeptides



Data from Table 3.6 for dipeptides with increasing R_1 chain-lengths and their methyl and benzyl

Figure 3.14 The effect of molecular surface area on IC_{50} for simple dipeptides



Data from Table 3.6 for dipeptides with increasing R_1 chain-lengths and their methyl and benzyl

The second possible conclusion for the patterns in IC_{50} values was that an increase in the size of the molecule reduces affinity for the transporter until a certain size is reached where affinity increases (and IC_{50} decreases). A measure of the size of a molecule is molecular surface area. Figure 3.14 (above) uses the molecular surface area data from Table 3.6 (above) to examine the relationship between IC_{50} and molecular surface area.

The data for the carboxylic acids and methyl esters show an increasing IC_{50} value until the surface area reaches 245-260. This data implies an optimum size of molecule. The results for the benzyl esters are on the right of the figure as they each contain a large bezene ring. The benzyl esters are never small enough to provide more information about the 245-260 range. The benzyl esters still have the increasing and then decreasing IC_{50} values which means that the pattern is independent of molecular surface area.

The data in Table 3.6 was analysed using the SPSS statistical computer programme. The result of the analysis is shown in Table 3.7 below.

Table 3.7 Pearson Correlation between IC_{50}, cLogP and Molecular Surface Area (MSA) for simple dipeptides

		IC_{50}	cLogP	MSA
IC_{50}	Pearson Correlation Significance p	1		
cLogP	Pearson Correlation Significance p	-0.240 0.389	1	
MSA	Pearson Correlation Significance p	-0.185 0.510	0.968 <0.001	1

Boxes shaded blue indicate a significant ($p<0.05$) relationship

Table 3.7 shows that for the 15 dipeptides in Table 3.6, cLogP significantly ($p<0.001$) correlated with Molecular Surface Area but IC_{50} did not significantly correlate with either cLogP ($p=0.389$) or Molecular Surface Area ($p=0.510$). Linear regression analysis of the data from Table 3.6 to predict IC_{50} derived Equation 3.1 below, which has large standard deviations (total ± 6.17) and was therefore not a good model.

Equation 3.1 Linear regression of IC_{50}, cLogP and Molecular Surface Area (MSA) for simple dipeptides

Predicted IC_{50} = [-2.861(±6.053)] + [-1.037(±1.182)*cLogP] + [0.0167(±0.025)*MSA]

Predicted IC_{50} will be ±6.17

A third hypothesis about the pattern of IC_{50} values relates to the "width" of the molecule. The dipeptides are 3-dimensional objects but can only bind to the transporter with one face. The width of this face is approximately the distance from the R_1 group to the R_3 group. Lengthening the R_1 side-chain by the addition of a methyl group will increase the width by the length of a carbon-carbon bond. The methyl esters will be the width of the equivalent carboxylic acid plus the length of an oxygen-carbon bond (as the R_3 group is –O-CH_3 instead of –OH). The benzyl esters will be the width of the equivalent carboxylic acid plus the length of an oxygen-carbon bond plus the length of a carbon-carbon bond plus the diameter of a benzene ring (as the R_3 group is –O-CH_2-C_6H_6 instead of –OH). The highest IC_{50} value for the carboxylic acids is the four-carbon side-chain (Nva-Pro). The width of this molecule will be similar to the three-carbon side-chain on a methyl ester (Abu-Pro OMe) which also has the largest IC_{50}. The largest IC_{50} for the benzyl esters has a two-carbon side-chain (Ala-Pro OBzl). The width of this molecule will be approximately the same as for Nva-Pro and Abu-Pro OMe. The variations in IC_{50} seen in Table 3.6 may therefore be due to the fact that a molecule of a certain width will have a low affinity and high IC_{50} value. More work needs to be performed to prove this hypothesis such as calculating the width of the molecules presented here. Until then, Key Finding 1a can be modified to Key Finding 1b.

Key Finding 1b: Increasing the side-chain length of dipeptides at the R_1 position increases IC_{50} until the chain reaches a certain length (dependent on an unknown factor but not cLogP or molecular surface area), at which point the IC_{50} decreases. This critical length may depend on the width of the dipeptide.

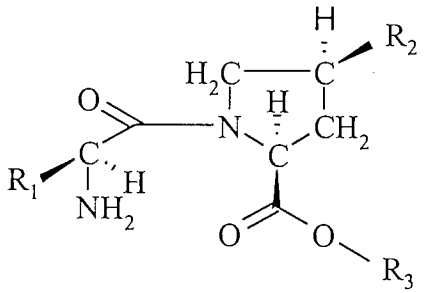
In summary, the sequential addition of a methyl group to the R_1 position to create a series of dipeptides and the esterification of the R_3 carboxylic acid to create methyl and benzyl esters, resulted in fifteen dipeptide derivative that were studies. The first key finding was that increasing the chain-length of the R_1 group increased IC_{50} up to a certain length. This

length is independent of cLogP and molecular surface area but may be dependent on the width of the molecules. The second finding was that the methyl and benzyl esters had different effects to their parent carboxylic acids implying that they were chemically stable. The final finding was that benzyl esters were equal or better inhibitors than the parent carboxylic acids and that the methyl esters were the worst.

3.3.3.2.3 The effect of dipeptides containing a sulphur atom in the R_1 side-chain

Changing groups at the R_1 position resulted in changing the side-chain of the dipeptide X-L-Pro. Table 3.8 below shows the IC_{50}s for dipeptide derivatives containing a sulphur atom and compares them with related hydrocarbon side-chains.

Table 3.8 Effect of sulphur-containing dipeptides on IC_{50}



When R_2 = H

Side chain	R_3 = H	R_3 = Me	R_3 = Bzl
R_1 =	Mean IC_{50} (±SD)	Mean IC_{50} (±SD)	Mean IC_{50} (±SD)
Alanyl (CH_3)	0.487 (0.042)	1.798 (1.030)	1.829 (0.320)
Abutyl (CH_2CH_3)	0.779 (0.180)	5.268 (2.679)	0.438 (0.132)
Cysteinyl (CH_2SH)	3.596 (0.551)	1.686 (0.100)	1.802 (0.113)
Methionyl (CH_2SCH_3)	2.637 (0.307)	0.415 (0.060)	0.218 (0.023)
Cysteinyl(S-benzyl) ($CH_2SC_6H_6$)	0.655 (0.106)	1.274 (0.683)	0.545 (0.229)

The first point to note is the poor solubility of these compounds and therefore the cysteinyl derivatives were dissolved in 2% DMSO to keep them in solution for testing. Figure 3.10 (the effect of DMSO on Gly-[^{3}H]L-Pro uptake) proved that this concentration should not affect uptake and so the results were retained and examined.

For the series of parent dipeptides (*i.e.* when R_3 = H), the addition of a methyl group to an alanyl sidechain (alanyl to abutyl) increased the IC_{50} but not significantly ($p>0.05$) but the addition of a thiol group to an alanyl side-chain (alanyl to cysteinyl) increased the IC_{50} significantly ($p<0.001$). Adding a methyl group to this thiol group (cysteinyl to methionyl) reduced the IC_{50} significantly ($p<0.05$) but the addition of a benzyl group to this thiol group (cysteinyl to cysteinyl(S-benzyl)) reduced the IC_{50} more significantly ($p<0.001$). This is summarised in Key Finding 4 below.

Key Finding 4: For the parent dipeptides, the addition of a thiol group to the R_1 side-chain resulted in a significant increase in IC_{50}. The addition of a methyl or benzyl group to the thiol group significantly reduced the IC_{50}.

The pattern for the methyl esters (where R_3 = CH_3) is less clear-cut due to the larger uncertainties in the estimates of IC_{50}. The addition of a thiol group to an alanyl side-chain (alanyl to cysteinyl) had no effect on IC_{50} ($p>0.05$). Adding a methyl group or a benzyl group to this thiol group (cysteinyl to methionyl or cysteinyl to cysteinyl(S-benzyl)) reduced the IC_{50} but not significantly ($p>0.05$). These results disagreed with Key Finding 4.

For the benzyl esters, the addition of a thiol group to an alanyl sidechain (alanyl to cysteinyl) had no effect on IC_{50} ($p>0.05$). Adding a methyl group or a benzyl group to this thiol group (cysteinyl to methionyl or cysteinyl to cysteinyl(S-benzyl)) significantly ($p<0.001$) reduced the IC_{50}. These results also disagreed with Key Finding 4.

In summary, the addition of a thiol group to an alanyl sidechain (alanyl to cysteinyl) had no effect on the methyl or benzyl esters but significantly increased the IC_{50} of the parent dipeptide. Cysteinyl dipeptides had very high IC_{50}s but these were reduced by the addition of other groups to the sulphur residue (significantly for the parent dipeptide derivative and benzyl ester, not ,significantly of the methyl esters). The addition of a methyl group (cysteinyl to methionyl) reduced IC_{50} in every case. The addition of a benzyl group (cysteinyl to cysteinyl(S-benzyl)) had a larger decrease in IC_{50} than the addition of a methyl group for the parent compounds but not for the esters. Key Finding 4 was therefore modified to Key Finding 4a.

Key Finding 4a: The addition of a thiol group to the R_1 side-chain increased the IC_{50} of the parent dipeptides but not for their methyl or benzyl esters. The addition of a methyl or benzyl group to the thiol group reduced the IC_{50}.

An ANOVA analysis of each row of Table 3.7 found significant ($p<0.05$) differences between results for the parent carboxylic acids and their esters for every row except for the cysteinyl(S-benzyl) esters. This lack of significance is probably due to the large uncertainty in the estimate of the IC_{50} for the methyl ester. The results support Key Finding 2: that the methyl and benzyl esters had significantly different results to the parent carboxylic acids implying that they are stable. However, using HPLC to measure the formation of the relevant acid as the ester hydrolyses is the only method to prove the stability.

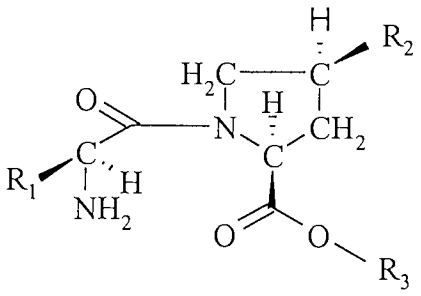
A previous finding (Key Finding 3) was that the R_3 benzyl esters were better than or equal to the parent acids as inhibitors and that the R_3 methyl esters were the worst inhibitors. The data presented in Table 3.8 shows that, in general for the sulphur containing side-chains, the benzyl esters had IC_{50}s lower than that for the parent dipeptide. The results for the methyl esters were more varied with lower IC_{50}s for cysteinyl and methionyl but a significantly higher IC_{50} for cysteinyl(S-benzyl). More results needed to be examined before Key Finding 3 was altered.

3.3.3.2.4 The effect of dipeptides containing an oxygen atom in the R_1 side-chain

As noted previously, changing groups at the R_1 position resulted in a changed side-chain of the dipeptide X-L-Pro. Table 3.9 below shows the IC_{50}s for dipeptide derivatives containing an oxygen atom (seryl) and compares them with a related hydrocarbon side-chain (alanyl).

The addition of an oxygen atom (alanyl to seryl) did not affect the IC_{50}s in the parent dipeptide or methyl ester ($p>0.05$) however it significantly ($p<0.05$) reduced the IC_{50} of the benzyl ester. The addition of the oxygen atom increased the cLogP of the parent acid and methyl ester by a small amount (0.2) but decreased the cLogP of the benzyl ester by a large amount (0.79) reducing its hydrophobicity.

Table 3.9 Effect of an oxygen atom in the R_1 sidechain of a dipeptide on IC_{50} and cLogP



When R_2 = H

Side chain	R_3 = H	R_3 = Me	R_3 = Bzl
R_1 =	Mean IC_{50} (±SD) *cLogP*	Mean IC_{50} (±SD) *cLogP*	Mean IC_{50} (±SD) *cLogP*
Alanyl (CH_3)	0.487 (0.042) *-0.84*	1.798 (1.030) *-0.81*	1.829 (0.320) *1.09*
Seryl (CH_2OH)	0.723 (0.106) *-0.63*	2.156 (0.184) *-0.60*	0.431 (0.040) *0.30*

The previous analysis of Table 3.6 found the IC_{50} for Ala-Pro Bzl was significantly higher than the other benzyl esters and that more studies needed to be performed. If the results for the benzyl esters are excluded then the main finding was Key Finding 5 below.

Key Finding 5: The addition of an oxygen atom to the R_1 side-chain caused a small increase in cLogP but no significant effect on IC_{50}

The results for the seryl side-chains follow the pattern seen in Table 3.3. The IC_{50} for the methyl ester is significantly higher than the parent dipeptide ($p<0.001$) and the benzyl ester has a lower IC_{50} (but not significant, $p>0.05$).

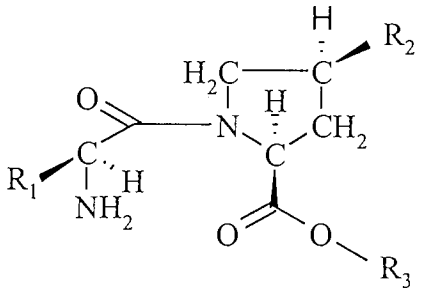
An ANOVA analysis of the results for the Ser-Pro derivatives found significant ($p<0.0001$) differences between results for the parent carboxylic acid and the two esters. The results support Key Finding 2: that the methyl and benzyl esters had significantly different results to the parent carboxylic acids implying that they are stable. The analysis also found that the benzyl IC_{50} was significantly ($p<0.05$) lower than the parent acid IC_{50} and the methyl IC_{50} was significantly ($p<0.001$) higher than the parent acid IC_{50}. This result supported

Key Finding 3 that the R_3 benzyl esters were better than or equal to the parent acids as inhibitors and that the R_3 methyl esters were the worst inhibitors.

3.3.3.2.5 The effect of dipeptides containing a nitrogen atom in the R_1 side-chain

Table 3.10 below shows the IC_{50}s for dipeptide derivatives containing a nitrogen atom (lysyl) and compares them with the nearest related hydrocarbon derivatives (norleucyl).

Table 3.10 Effect of a nitrogen atom in the R_1 sidechain of a dipeptide on IC_{50} and cLogP



When R_2 = H

Side chain	R_3 = H	R_3 = Me	R_3 = Bzl
R_1 =	Mean IC_{50} (±SD) *cLogP*	Mean IC_{50} (±SD) *cLogP*	Mean IC_{50} (±SD) *cLogP*
Norleucyl ($CH_2CH_2CH_2CH_3$)	0.299 (0.027) *0.42*	0.786 (0.071) *0.45*	0.438 (0.044) *2.35*
Lysyl ($CH_2CH_2CH_2CH_2NH_2$)	1.111 (0.427) *-1.08*	3.058 (0.322) *-1.04*	2.853 (0.708) *0.85*

The change from a norleucyl to a lysyl side-chain (CH_3 to CH_2NH_2) resulted in a significant increase in IC_{50} for the parent dipeptide ($p<0.05$), methyl ester ($p<0.001$) and benzyl ester ($p<0.01$). Norleucyl was a poor comparator for lysyl as both a methyl group and an amino group had to be added to make lysyl. However, it was concluded that lysyl dipeptides are poor inhibitors. In each case, the addition of the methylamino group reduced the IC_{50} by 1.5. This reduced hydrophobicity may result in a reduced affinity and hence the larger IC_{50}.

Key Finding 6: Lysyl-proline dipeptides were poor inhibitors. This may be due to their chain-length or the presence of an amino group at the end of the R_1 side-chain affecting the hydrophobicity of the dipeptide.

An ANOVA analysis of the results for the Lys-Pro derivatives found significant ($p<0.001$) differences between results for the parent carboxylic acid and the two esters. The results support Key Finding 2: that the methyl and benzyl esters had significantly different results to the parent carboxylic acids implying that they are stable.

The analysis also found no significant difference between the IC_{50} for lysyl methyl and benzyl esters, however both were significantly ($p<0.05$) higher than the parent dipeptide IC_{50}. This result contrasts with Key Finding 3 that the R_3 benzyl esters were better than or equal to the parent acids as inhibitors and that the R_3 methyl esters were the worst inhibitors.

This contrast may be due to the effect of a positive charge. The parent dipeptide has two amino groups (both positively charged) and one carboxylate group (negatively charged) and so has an overall charge of plus one and not a very good IC_{50}. Both esters have the two amino groups but lack the carboxylate group. They have an overall charge of plus two and larger IC_{50} values. These results led to Key Finding 7.

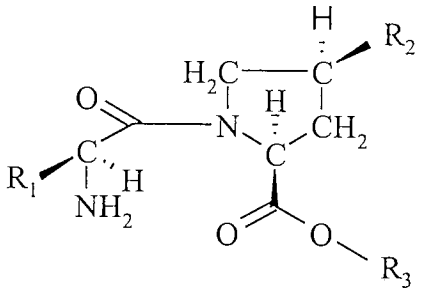
Key Finding 7: A dipeptide with a net molecular charge of +1 had a poor affinity for the transporter. Dipeptide derivatives with a charge of +2 had an even worse affinity.

3.3.3.2.6 The effect of dipeptides containing a carboxyl group in the R_1 side-chain

Table 3.11 below shows the IC_{50}s for dipeptide derivatives containing a carboxyl group and compares them with related hydrocarbon side-chains. The compounds were grouped to show the effect of changing a methyl group (CH_3) to a carboxylate group (COOH). Changing the abutyl group to an aspartyl group led to a 1.09 reduction in cLogP but had no significant ($p>0.05$) effect on the parent acid or benzyl ester but significantly ($p<0.05$) reduced the IC_{50} of the methyl ester. Changing the norvalyl group to a glutamyl group led to a 1.23 reduction in cLogP that significantly ($p>0.01$) reduced the IC_{50} of the parent acid, had no effect ($p>0.05$) on the methyl ester, and significantly ($p<0.01$) increased the IC_{50} of the benzyl ester. The range of effects meant that no conclusion could be made.

Key Finding 8: Changing an R_1 methyl group to a carboxylic group produced a drop in cLogP and a range of effects on IC_{50}.

Table 3.11 Effect of a carboxyl group in the R_1 sidechain of a dipeptide on IC_{50} and cLogP



When R_2 = H

Side chain	R_3 = H	R_3 = Me	R_3 = Bzl
R_1 =	Mean IC_{50} (±SD) *cLogP*	Mean IC_{50} (±SD) *cLogP*	Mean IC_{50} (±SD) *cLogP*
Abutyl (CH_2CH_3)	0.779 (0.180) *-0.37*	5.268 (2.679) *-0.34*	0.438 (0.132) *1.56*
Aspartyl (CH_2COOH)	1.066 (0.068) *-1.46*	1.282 (0.088) *-1.43*	0.856 (0.461) *0.47*
Aspartyl (o-benzyl) ($CH_2COOC_6H_6$)	3.941 (1.207) *0.47*	0.748 (0.145) *0.50*	0.986 (0.073) *2.40*
Norvalyl ($CH_2CH_2CH_3$)	1.208 (0.136) *0.02*	1.161 (0.131) *0.05*	0.686 (0.054) *1.95*
Glutamyl (CH_2CH_2COOH)	0.483 (0.107) *-1.21*	1.101 (0.372) *-1.18*	1.094 (0.101) *0.72*
Glutamyl (o-benzyl) ($CH_2CH_2COOC_6H_6$)	1.962 (0.771) *0.72*	0.294 (0.074) *0.75*	0.157 (0.027) *2.65*

The compounds were then grouped to show the effect of changing a carboxylate group (COOH) to a benzyl ester ($COOC_6H_6$). Changing the aspartyl group to an aspartyl (O-benzyl) group led to a 1.93 increase in cLogP that significantly ($p<0.01$) increased the IC_{50} of the parent acid, significantly reduced the IC_{50} of the methyl ester and had no significant ($p>0.05$) effect on the benzyl ester. Changing the glutamyl group to a glutamyl (O-benzyl) group led to a 1.93 increase in cLogP that significantly ($p>0.05$) increased the IC_{50} of the parent acid and significantly reduced the IC_{50} of the methyl ($p<0.05$) and benzyl ($p<0.001$). The range of effects again meant that no conclusion could be made so Key Finding 8 was adapted as below.

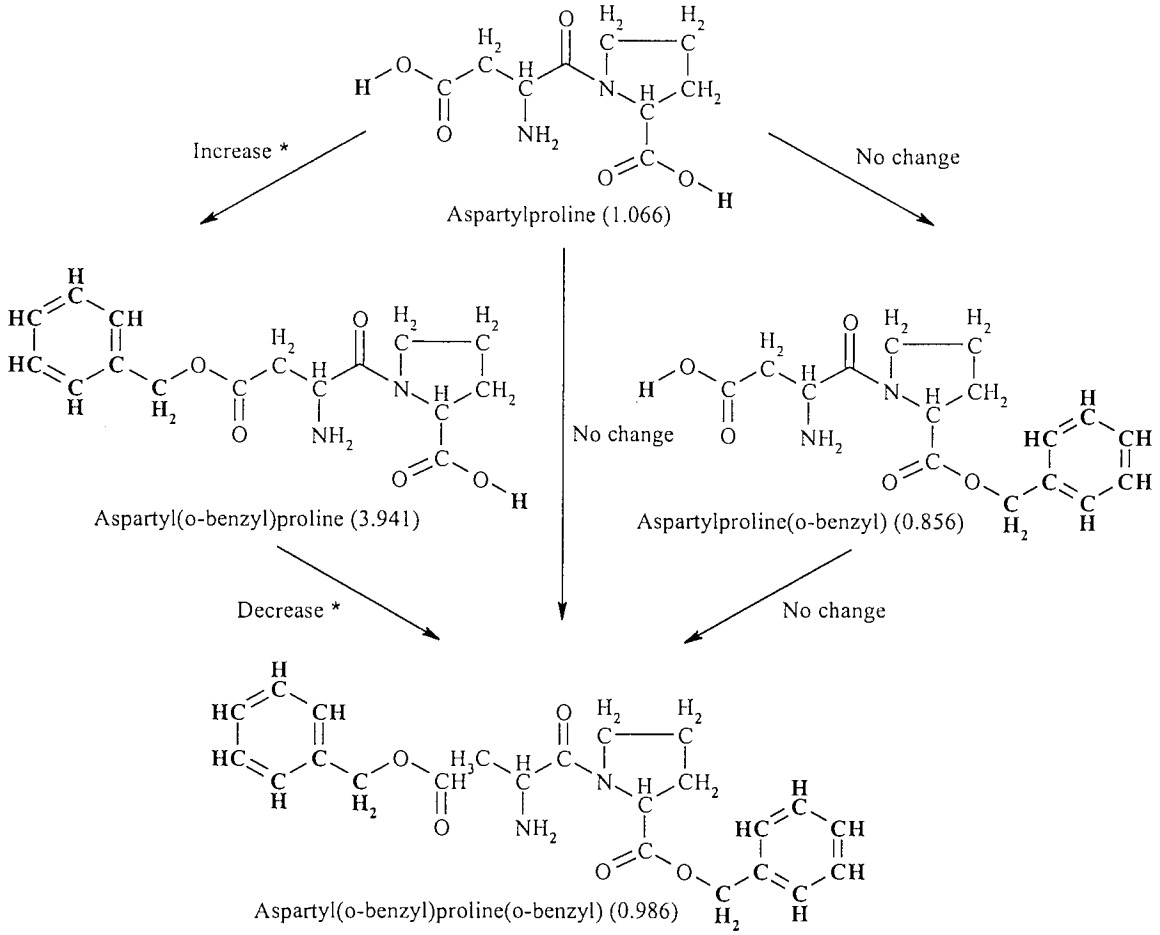
Key Finding 8a: Changing an R_1 methyl group to a carboxylate group produced a drop in cLogP and a range of effects on IC_{50}. Adding a benzyl group to the carboxylate group produced another range of effects on IC_{50}.

An ANOVA analysis of the results in Table 3.11 found significant ($p<0.01$) differences between results for the parent carboxylic acid and the two esters for all dipeptides except the aspartyl-proline derivatives. This exception may be due to the large error in estimating the affinity of the benzyl ester. In general, the results support Key Finding 2: that the methyl and benzyl esters had significantly different results to the parent carboxylic acids implying that they are stable.

Key Finding 3 was that the R_3 benzyl esters were better than or equal to the parent acids as inhibitors and that the R_3 methyl esters were the worst inhibitors. The effect of changing R_3 for aspartyl and glutamyl residues is different. There was no significant difference in the IC_{50}s for the methyl and benzyl esters in either case. For aspartyl, the esters had no significant difference in IC_{50} compared to the parent dipeptide. For glutamyl, the esters had significantly ($p<0.05$) higher IC_{50}s than the parent dipeptide. For aspartyl(O-benzyl) and glutamyl(O-benzyl), the esters had significantly ($p<0.01$) lower IC_{50}s than the parent dipeptide. This range of results could be due to the overall charge on the molecule.

The effect of creating benzyl esters of aspartylproline on IC_{50} is shown diagrammatically in Figure 3.15 below. The creation of a C-terminal benzyl ester (to make aspartylproline(O-benzyl)) had no significant effect on IC_{50}. The addition of a second benzyl group, this time to the aspartyl acid (to make aspartyl(O-benzyl)proline(O-benzyl) also had no significant effect on IC_{50}. It was concluded that the addition of two large hydrophobic groups to aspartylproline had no significant effect on IC_{50}. However the results were different if the esters were formed in a different order. The addition of a benzyl group to the aspartyl acid (to make aspartyl(O-benzyl)proline) significantly increased IC_{50}. The addition of the second benzyl group, this time to the prolyl acid (to make aspartyl(O-benzyl)proline(O-benzyl)) significantly reduced IC_{50}. It was then concluded that the addition of two large hydrophobic groups to aspartylproline had no significant effect on IC_{50} but the addition of a single benzyl group significantly increased IC_{50}, and the addition of a second benzyl group countered the effect. These findings are summarised in Key Finding 9 below.

Figure 3.15 Effect on IC_{50} of the addition of benzyl groups to Aspartylproline



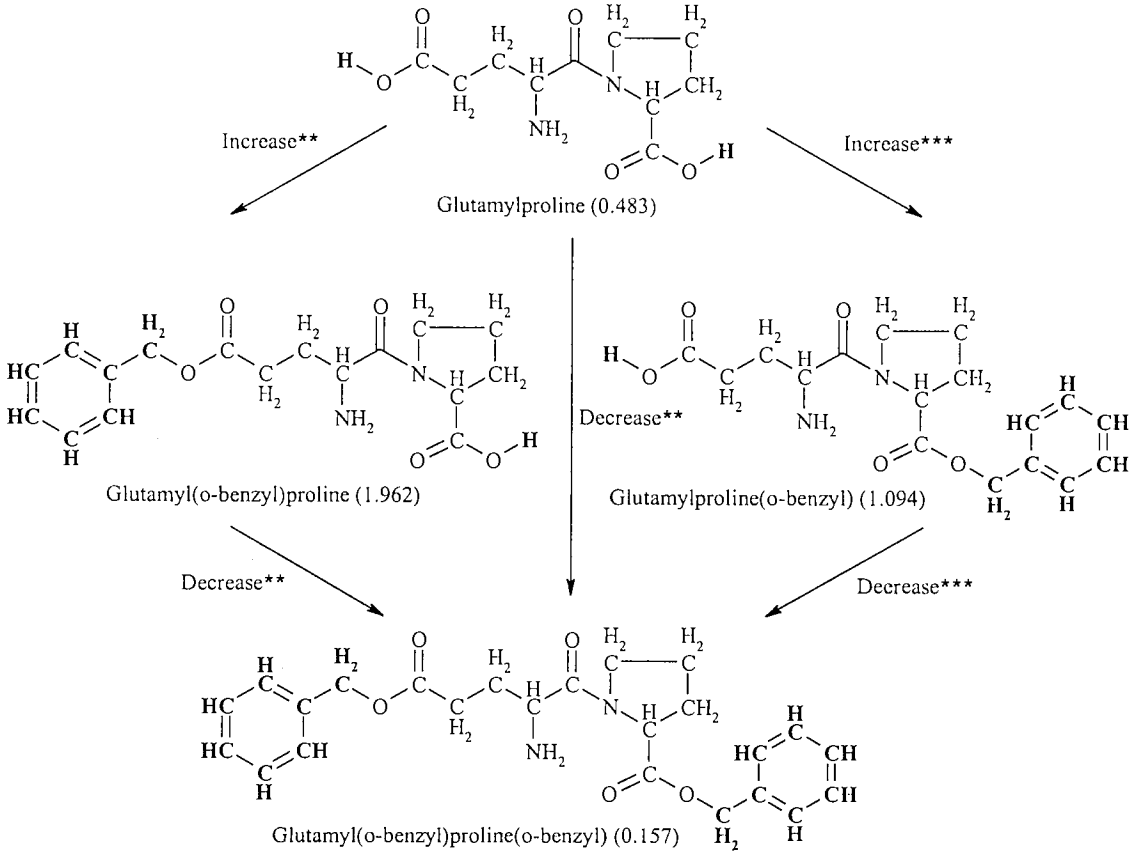
Number in brackets equals IC50 in mM.

* denotes significant changes in IC50 - * = $p<0.05$

Key Finding 9: The simultaneous addition of a benzyl group to both carboxyl groups in aspartylproline had no effect on IC_{50}. However, the esterification of the aspartyl carboxyl increased IC_{50} and the sequential esterification of the proline carboxyl decreased IC_{50}.

The effect of creating benzyl esters of glutamylproline on IC_{50} is shown diagrammatically in Figure 3.16 below. The creation of the C-terminal ester (to make glutamylproline(O-benzyl)) significantly ($p<0.001$) increased IC_{50}. The addition of a second benzyl group, this time to the glutamyl acid (to make glutamyl(O-benzyl)proline(O-benzyl)) significantly ($p<0.001$) reduced IC_{50}, below that of glutamylproline. It was concluded that the addition of two large hydrophobic groups to glutamylproline significantly reduced IC_{50}. If the order of addition of the benzyl groups was reversed, the same pattern of increased and the decreased IC_{50} was seen. Key Finding 9 was therefore altered as below.

Figure 3.16 Effect on IC_{50} of the addition of benzyl groups to Glutamylproline



Number in brackets equals IC50 in mM.

* denotes significant changes in IC50 - ** = $p<0.01$, *** = $p<0.001$

Key Finding 9a: The simultaneous addition of a benzyl group to both carboxyl groups had no effect on IC_{50} for aspartylproline but significantly reduced the IC_{50} of glutamylproline. In general, the esterification of one carboxyl group increased IC_{50} and the sequential esterification of the other carboxyl decreased IC_{50}.

3.3.3.2.7 The effect of charged atoms on IC_{50}

For both aspartylproline and glutamylproline, the addition of one benzyl group increased IC_{50}, and the addition of a second benzyl group countered this. The parent dipeptides have one amino group (positive) and two carboxylate groups (negative) resulting in an overall charge of minus one and a good IC_{50}. The monoesters have one amino group and one carboxylate group resulting in an overall charge of zero and a larger IC_{50}. The diesters have one amino group resulting in an overall charge of plus one and the lowest IC_{50}. These results imply that the reduction in IC_{50} is due to either the positive charge or the increase in

hydrophobicity. The results for the lysylproline derivatives are compared to the glutamylproline derivatives in Table 3.12 below.

Table 3.12 Effect of net charge and cLogP on IC_{50}

Name	Glu-Pro[a]	Glu-Pro(O-bzl)[b]	Glu(O-bzl)-Pro(O-bzl)[c]	
IC50 (±SD)	0.483 (0.107)	1.094 (0.101)	0.157 (0.027)	
cLogP	*-1.21*	*0.72*	*2.65*	
Net Charge	-1	0	+1	

Name			Lys-Pro[d]	Lys-Pro(O-bzl)[e]
IC50 (±SD)			1.111 (0.427)	2.853 (0.708)
cLogP			*-1.08*	*0.85*
Net Charge			+1	+2

[a] Glu-Pro – Glutamylproline,

[b] Glu-Pro(O-bzl) – Glutamylproline(O-benzyl),

[c] Glu(O-bzl)-Pro(O-bzl) – Glutamyl(O-benzyl)proline(O-benzyl),

[d] Lys-Pro – Lysylproline,

[e] Lys-Pro(O-bzl) – Lysylproline(O-benzyl)

The actual net charge on the molecule will depend on the molecular pI and pH of the solution it is in. The net charges will not be exactly –1, 0, +1 and +2 but the derivatives can be grouped under the headings of –1, 0, +1 and +2.

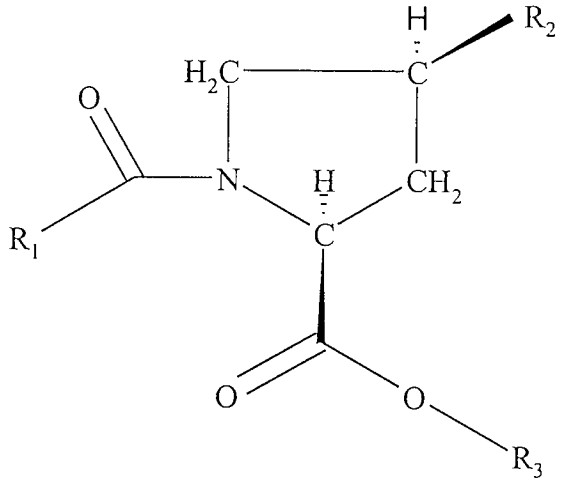
There are no clear conclusions to be drawn from Table 3.12, as there are a large number of variables such as charge and hydrophobicity. However, for glutamylproline and lysylproline derivatives, the best inhibitors have a charge of plus one. This could mean that there is a residue with a negatively charged side-chain near the active site of the transporter.

Key Finding 10: For glutamylproline and lysylproline, the best inhibitors had a charge of +1. This may have been due to a negatively charged sidechain near the active site of the transporter.

3.3.3.2.8 The effect of non-dipeptide residues on IC_{50}

Table 3.13 below shows the IC_{50}s for the non-dipeptide derivatives tested. It states that adipic(O-methyl)proline(O-methyl) had an infinite IC_{50}. This result is explained below.

Table 3.13 Effect of non-dipeptide residues



When R_2 = H

Side chain	R_3 = H	R_3 = Me	R_3 = Bzl
R_1 =	Mean IC_{50} (±SD)	Mean IC_{50} (±SD)	Mean IC_{50} (±SD)
Sarcosyl (CH_2NHCH_3)	0.377 (0.073)	3.161 (1.804)	0.583 (0.136)
Suboic(O-ethyl) ($C_3H_6COOC_2H_5$)	Not tested	2.815 (1.618)	1.331 (0.290)
Adipic(O-methyl) ($C_5H_{10}COOCH_3$)	7.503 (3.444)	Infinite	2.319 (0.728)
Pimoic(O-ethyl) ($C_6H_{12}COOC_2H_5$)	2.269 (0.405)	2.336 (0.548)	1.624 (0.678)

The measured uptake of Gly-[^{3}H]L-Pro in the presence of 1mM adipic(O-methyl)proline(O-methyl) was 96.8±2.9% of the mean control uptake. Some wells had an uptake just over 100% of the mean control uptake and therefore had very large negative IC_{50}s. Some wells had an uptake just under 100% of the mean control uptake and therefore had very large positive IC_{50}s. The mean (±SD) of the IC_{50}s of the wells was –21.2(±92.6) mM. This value was not used, as the standard deviation would cover all other calculated IC_{50}s. Adipic(O-methyl)proline(O-methyl) was not a good inhibitor so it was given a very

large but undefined IC_{50} *i.e.* infinite. This value gives an idea of how bad an inhibitor it was but was not used for any significance testing.

An ANOVA analysis of the results in Table 3.13 found no significant ($p>0.05$) differences between results for the parent carboxylic acid and the two esters for all non-dipeptides except for sarcosylproline where the methyl ester had a significantly ($p<0.01$) larger IC_{50} than that for the parent acid. In general, the results do not support Key Finding 2: that the methyl and benzyl esters had significantly different results to the parent carboxylic acids implying that they are stable. This limited the conclusions that could be drawn for these compounds.

Key Finding 2a: For the dipeptide derivatives, the methyl and benzyl esters had significantly different results to the parent carboxylic acids implying that they are stable. This was not true for the non-dipeptide derivatives.

Sarcosylproline is structurally very similar to alanylproline and it is therefore not surprising that it had a low IC_{50}. As stated above, its methyl ester had a significantly higher IC_{50} while the IC_{50} of the benzyl ester was not significantly different to the parent acid. These results agreed with Key Finding 3, that the R_3 benzyl esters were better than or equal to the parent acids as inhibitors and that the R_3 methyl esters were the worst inhibitors. These results led to Key Finding 11 below.

Key Finding 11: Sarcosylproline had a low IC_{50} value and esterification at the R_3 position had the same effect as esterification at the R_3 position of dipeptides.

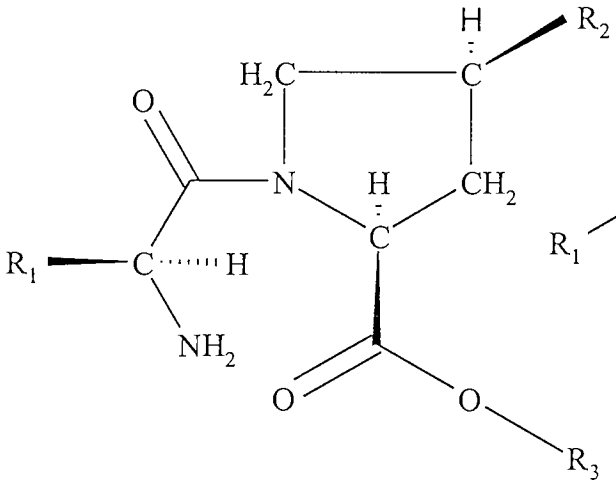
The other conclusion that was drawn was that suboic, adipic and pimoic acid derivatives had larger IC_{50} values than the dipeptide derivatives and their benzyl esters causing non-significant ($p>0.05$) decreases in IC_{50}.

Key Finding 12: The non-dipeptide derivatives, composed of esterified dicarboxylic acids joined to a proline residue, were inhibitors of uptake but were much less potent than the dipeptide derivates.

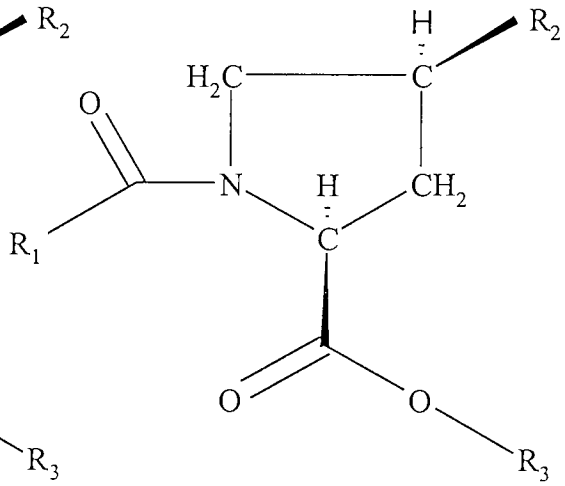
3.3.3.2.9 The effect of diphenylmethyl esters on IC_{50}

Changing groups at the R_3 position resulted in esterifying the prolyl carboxylate group of the dipeptides and non-dipeptides X-L-Pro (structure previously shown in Figure 3.12). Key Finding 3 was that the R_3 benzyl esters (R_3 = Bzl) were better than or equal to the parent acids (R_3 = H). The effects of diphenylmethyl esters (R_3 = dPhMe) were studied and compared with the effect of the other R_3 esters (Table 3.14 below).

Table 3.14 Effect of dibenzyl esters on IC_{50}



Dipeptide derivative



Non-dipeptide derivative

When R_2 = H

Side chain	R_3 = H	R_3 = Me	R_3 = Bzl	R_3 = dPhMe
R_1 =	Mean IC_{50} (±SD)	Mean IC_{50} (±SD)	Mean IC_{50} (±SD)	Mean IC_{50} (±SD)
DIPEPTIDE				
Methionyl (CH_2SCH_3)	2.637 (0.307)	0.415 (0.060)	0.218 (0.023)	1.851 (0.480)
Aspartyl (O-benzyl) ($CH_2COOC_6H_6$)	3.941 (1.207)	0.748 (0.145)	0.986 (0.073)	3.695 (0.733)
Glutamyl (O-benzyl) ($CH_2CH_2COOC_6H_6$)	1.962 (0.771)	0.294 (0.074)	0.157 (0.027)	1.105 (0.200)
NON-DIPEPTIDE				
Suboic (O-ethyl) ($C_3H_6COOC_2H_5$)	Not tested	2.815 (1.618)	1.331 (0.290)	3.956 (2.942)
Adipic (O-methyl) ($C_5H_{10}COOCH_3$)	7.503 (3.444)	Infinite	2.319 (0.728)	3.499 (1.329)
Pimoic (O-ethyl) ($C_6H_{12}COOC_2H_5$)	2.269 (0.405)	2.336 (0.548)	1.624 (0.678)	3.153 (0.666)

The reason for adipic(O-methyl)proline(O-methyl) having an infinite IC_{50} was explained previously in Section 3.3.3.2.8. An ANOVA analysis of the results in Table 3.14 found that for the dipeptide derivatives, there was a significant ($p<0.001$) difference between the IC_{50}s for the parent acid and three esters. These results support Key Finding 2a: that for the dipeptide derivatives, the different esters had significantly different results to the parent carboxylic acids implying that they were stable. An ANOVA analysis of the non-dipeptide results only found a significant ($p<0.05$) difference in the IC_{50} values of the pimoic(O-ethyl) esters. The lack of significance limited the conclusions that could be drawn for the non-dipeptide derivatives.

Table 3.3 is repeated below to show the different structures of the R_3 groups. The creation of diphenylmethyl esters from the parent acids (H to $CH(C_6H_6)_2$) only significantly ($p<0.05$) reduced the IC_{50} of methionylproline and had no significant effect on any other parent acid. The addition of a second benzyl group to a benzyl ester (from a benzyl ester to a diphenylmethyl ester) ($CH_2C_6H_6$ to $CH(C_6H_6)_2$) always resulted in an increase in IC_{50} (significantly for pimoic(O-ethyl) ($p<0.05$), glutamyl (O-benzyl) ($p<0.01$), aspartyl(O-benzyl) ($p<0.01$), and for methionyl ($p<0.001$)).

Table 3.3 Structures of different R_3 groups tested (for both sets of derivatives)

R_3 Structure	Residue Name
H	Carboxylic acid
CH_3	Methyl ester
$CH_2C_6H_6$	Benzyl ester
$CH(C_6H_6)_2$	Diphenylmethyl ester

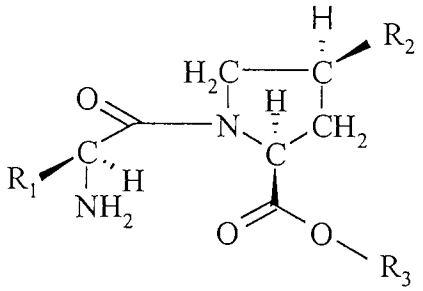
The addition of the extra benzyl group makes the molecule larger and more hydrophobic but increases the IC_{50} and therefore decreases affinity. This could mean that there are optimum ranges of molecular size and hydrophobicity. The diphenylmethyl esters of more dipeptides will need to be synthesised and tested before firm conclusions can be made.

Key Finding 13: The diphenylmethyl esters of dipeptides that were tested were worse inhibitors than benzyl esters.

3.3.3.2.10 The effect of changing the R_2 group on IC_{50}

Two different groups were tested at the R2 position. A hydrogen atom in this position resulted in a proline residue while a benzoxy group resulted in a benzoxyproline residue. Table 3.15 below shows the IC_{50}s for the benzoxyprolyl compounds tested and compares them with their prolyl equivalents.

Table 3.15 Effect of changing R_2 residues



When R_3 = Me

Side chain	R_2 = H	$R_2 = OCH_2C_6H_6$
R_1 =	Mean IC_{50} (±SD)	Mean IC_{50} (±SD)
Methionyl (CH_2SCH_3)	0.415 (0.060)	1.663 (0.571)
Aspartyl (CH_2COOH)	1.282 (0.088)	Infinite
Aspartyl (O-benzyl) ($CH_2COOC_6H_6$)	0.748 (0.145)	0.878 (0.218)
Glutamyl (CH_2CH_2COOH)	1.101 (0.372)	6.460 (3.242)
Glutamyl (O-benzyl) ($CH_2CH_2COOC_6H_6$)	0.294 (0.074)	1.520 (0.295)

Table 3.12 states that aspartylbenzoxyproline(O-methyl) had an infinite IC_{50}. The measured uptake was 103.9±3.6% of the mean control uptake. The wells had an uptake just over 100% of the mean control uptake and therefore have very large negative IC_{50}s. The mean of the IC_{50}s of the wells was –39.2(±37.0) mM. This value was not used as it is a negative IC_{50}. Aspartylbenzoxyproline(O-methyl) was not a good inhibitor so it was given a very large but not defined IC_{50} i.e. infinite. This value gives an idea of how good an inhibitor it is but was not used for any significance testing. It should also be noted that

there was a methyl ester in the R_3 position of all the compounds tested in Table 3.15. Key Finding 3 was that the R_3 benzyl esters are better than or equal to the parent acids as inhibitors and that the R_3 methyl esters are the worst inhibitors, although this slightly differed for the charged dipeptides, aspartylproline and glutamylproline, and their derivatives. In those cases, the methyl esters were as good at inhibiting as the benzyl esters.

The change from proline to benzoxyproline increased IC_{50} in every case. It was not a significant ($p>0.05$) increase for aspartyl(O-benzyl) or glutamyl, was a significant ($p<0.05$) change for methionyl and glutamyl(O-benzyl) but not measurable for aspartyl. An increase in IC_{50} means a decrease in affinity. The addition of the benzoxy group increased the size of the molecule and therefore the changed shape may have been less able to bind to the transporter.

Key Finding 14: The addition of a benzoxy group to the R_2 position decreased affinity for the transporter.

3.3.3.2.11 Summary of structure-activity relationships

The final versions of the key findings are listed below:

- Key Finding 1: Increasing the side-chain length of dipeptides at the R_1 position increases IC_{50} until the chain reaches a certain length (dependent on an unknown factor but not cLogP or molecular surface area), at which point the IC_{50} decreases. This critical length may depend on the width of the dipeptide.
- Key Finding 2: For the dipeptide derivatives, the methyl and benzyl esters had significantly different results to the parent carboxylic acids implying that they were stable. This was not true for the non-dipeptide derivatives.
- Key Finding 3: The R_3 benzyl esters are better than or equal to the parent acids as inhibitors and that the R_3 methyl esters are the worst inhibitors.
- Key Finding 4: The addition of a thiol group to the R_1 side-chain increased the IC_{50} of the parent dipeptides but not for their methyl or benzyl esters. The addition of a methyl or benzyl group to the thiol group reduced the IC_{50}.
- Key Finding 5: The addition of an oxygen atom to the R_1 side-chain caused a small increase in cLogP but no significant effect on IC_{50}

- Key Finding 6: Lysyl-proline dipeptides were poor inhibitors. This may be due to their chain-length or the presence of an amino group at the end of the R_1 side-chain affecting the hydrophobicity of the dipeptide.
- Key Finding 7: A dipeptide with a net molecular charge of +1 had a poor affinity for the transporter. Dipeptide derivatives with a charge of +2 had an even worse affinity.
- Key Finding 8: Changing an R_1 methyl group to a carboxylate group produced a drop in cLogP and a range of effects on IC_{50}. Adding a benzyl group to the carboxylate group produced another range of effects on IC_{50}.
- Key Finding 9: The simultaneous addition of a benzyl group to both carboxyl groups had no effect on IC_{50} for aspartylproline but significantly reduced the IC_{50} of glutamylproline. In general, the esterification of one carboxyl group increased IC_{50} and the sequential esterification of the other carboxyl decreased IC_{50}.
- Key Finding 10: For glutamylproline and lysylproline, the best inhibitors have a charge of +1. This may have been due to a negatively charged sidechain near the active site of the transporter.
- Key Finding 11: Sarcosylproline had a low IC_{50} value and esterification at the R_3 position had the same effect as esterification at the R_3 position of dipeptides.
- Key Finding 12: The non-dipeptide derivatives, composed of esterified dicarboxylic acids joined to a proline residue, were inhibitors of uptake but were much less potent than the dipeptide derivates.
- Key Finding 13: The diphenylmethyl esters of dipeptides that were tested were worse inhibitors than benzyl esters.
- Key Finding 14: The addition of a benzoxy group to the R_2 position decreased affinity for the transporter.

Figure 3.17 below shows these how these key findings relate to the structure of Gly-Pro. The first main area of investigation was the effect on affinity of uncharged dipeptide side-chains. Increasing the chain-length first reduced then increased affinity. This could be due to the increase in hydrophobicity. The presence of a free thiol group reduced affinity while methylating the group increased affinity. The presence of an oxygen atom in the side-chain had no effect on affinity. In summary, the uncharged derivatives with the strongest affinities had long side-chains and no free thiol group.

Figure 3.17 Summary of Key Findings for uptake *via* the dipeptide transporter

* Increasing side-chain length reduces then increases affinity

* A free thiol group reduces affinity

* Methylating a free thiol group increases affinity

* The presence of an oxygen atom has no effect on affinity

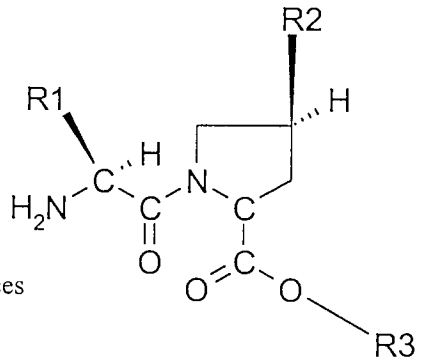
* Lysyl-proline derivatives have low affinity

* Carboxylating this group produces a range of effects on affinity

* Non-dipeptide derivatives have a low affinity

* Derivatives with a net charge of +1 have a reduced affinity while dipeptides with any other net charge have even more reduced affinities

* The presence of a benzoxy group reduces affinity



* Methyl and benzyl esters have different affinities compared to the parent dipeptide

* Benzyl esters have a larger affinity than the parent dipeptide

* Methyl esters have a lower affinity than the parent dipeptide

* Diphenylmethyl esters have a lower affinity than the benzyl esters

The second area of investigation was the effect of charged side-chains on affinity. Side-chains with a positive charge (lysyl-proline derivatives) had a low affinity for the transporter. Side-chains with a carboxylate group (*e.g.* aspartyl-proline derivatives) had a range of affinities. A review of all the charged derivatives found that the net charge on the derivative affected affinity. Derivatives with a net charge of +1 had a lower affinity than uncharged derivatives, however derivatives with other net charges had significantly lower affinities. It can be concluded that charged derivatives with the strongest affinities had a net charge of +1.

The third main area of investigation was studying the effect of modifying the α-carboxyl group. Benzyl esters in this position were found to have affinities higher or equal to the parent acids while methyl esters had lower affinities. Diphenylmethyl esters had lower affinities than benzyl esters. The creation of esters affects the net charge of the derivative and would be expected to reduce affinity (see above). This seems to be the case for the methyl esters. The benzyl esters increase the size and hydrophobicity of the molecules that would be expected to increase affinity. The increase in hydrophobicity appears to

overcome the effect of creating a charged derivative. It can be concluded that benzyl esters are a constructive method to remove a negative charge while increasing affinity.

In summary, the structure-activity relationships identified have informed the identification of the structure of optimal inhibitors of the dipeptide transporter. For uncharged derivatives, it is to have long side-chains and no free thiol groups. For charged derivatives, it is to have a net charge of +1. If carboxylate groups need to be modified, benzyl esters abolish the charge but increase the affinity for the transporter. Further studies can use these optimal inhibitor structures to lead to the identification of optimal transport substrates to inform drug delivery.

3.3.3.3 Statistical analysis of all results

In addition to structure-activity relationships, molecular parameters can be calculated and used to identify any correlation with affinity (IC_{50}). Twelve molecular parameters were derived, using the CAChe and Tsar computer programmes, for the sixty-four compounds that had been tested. The twelve parameters are described in Table 3.16 below. In addition to the molecular parameters, five parameters (molecular weight, cLogP, molar refractivity, molecular surface area, and dipole moment) were calculated for the different structures in the R_1, R_2, and R_3 positions.

Table 3.16 Calculated molecular parameters and their brief descriptions

Molecular Weight (MW)	The sum of the atomic weights of all the constituent atoms
cLogP	The Log octanol/water coefficient – a measure of hydrophobicity
Molar Refractivity (MR)	Related to the molecular size and polarity
Molecular Surface Area (MSA)	The surface area of the molecule
Dipole moment (DIP)	The polarity of the molecule – calculated using CACHe
Calculated dipole (CDIP)	The polarity of the molecule – calculated using TSAR
Steric energy (Steric)	The sum of the energies involved in bond stretching, bond angle bending, and van der Waals and electrostastic interactions
HOMO	Highest Occupied Molecular Orbit – related to electron donation
LUMO	Lowest Unoccupied Molecular Orbit – related to electron accepting
Heat of Formation (HoF)	The theoretical energy needed to create the molecule from elements
Donor	The number of hydrogen atoms attached to N, O or S
Accept	The number of H-bond acceptor groups (N, O, S, F, Cl, Br, I)

3.3.3.3.1 Correlation of molecular parameters with IC_{50}

A Pearson correlation of all the parameters for all the molecules was performed (using SPSS) and the results are shown in Table 3.17 below. Significant ($p<0.05$) correlations are shown in bold. Only two parameters significantly correlated with IC_{50}, Heat of Formation ($p=0.032$) and Accept ($p=0.048$). Heat of Formation also significantly correlated with parameters relating to molecular size (MW and MSA), hydrophobicity (LogP), polarity (MR) and both the number of hydrogen-bond donor and acceptor groups (HOMO, LUMO, Donor and Accept).

Seven parameters significantly correlated with Heat of Formation but not significantly with IC_{50}. This may mean that the seven parameters (molecular weight, Log P, molar refractivity, molecular surface area, HOMO, LUMO and number of hydrogen-bond donors) do affect IC_{50} but they cause such minor changes that it was only when they were taken together that they caused a significant effect.

The larger dipeptides tended to have benzene rings so it was no surprise that molecular weight, Log P, molar refractivity and molecular surface area all correlated. Key Finding 3 was that the R_3 benzyl esters were better inhibitors. The finding therefore agrees with the statistical analysis.

Table 3.17 Pearson correlations between molecular parameters

	IC50	*MW*	*LogP*	*MR*	*MSA*	*DIP*	*CDIP*	*Steric*	*HOMO*	*LUMO*	*HoF*	*Donor*	*Accept*
IC50	1.000												
MW	0.062	1.000											
LogP	-0.020	**0.883**	1.000										
MR	0.027	**0.994**	**0.915**	1.000									
MSA	0.036	**0.986**	**0.918**	**0.991**	1.000								
DIP	-0.199	0.119	0.117	0.127	0.197	1.000							
CDIP	0.031	0.138	0.054	0.119	0.134	**0.614**	1.000						
Steric	-0.163	**0.720**	**0.638**	**0.754**	**0.756**	0.045	-0.088	1.000					
HOMO	-0.175	-0.040	-0.043	0.017	0.007	0.102	-0.031	0.223	1.000				
LUMO	0.048	-0.035	0.106	-0.038	-0.022	0.076	-0.001	-0.189	**-0.547**	1.000			
HoF	**-0.269**	**-0.722**	**-0.536**	**-0.651**	**-0.663**	-0.088	-0.241	-0.174	**0.513**	**-0.276**	1.000		
Donor	-0.149	**-0.447**	**-0.621**	**-0.477**	**-0.491**	-0.173	-0.030	-0.068	0.012	**-0.262**	**0.313**	1.000	
Accept	**0.248**	**0.519**	0.162	**0.428**	**0.450**	0.028	0.211	0.168	**-0.499**	0.199	**-0.855**	0.084	1.000

See table 3.16 for abbreviations

A Pearson correlation, of 13 parameters for all of the 64 compounds tested, was performed. Significant ($p<0.05$) correlations are shown in bold and highlighted in blue.

Four parameters relating to H-bond formation were analysed (HOMO, LUMO, Donor and Accept). They significantly cross-correlated except for HOMO and Donor, and LUMO and Accept, however only Accept correlated with IC_{50}. Accept was described in Table 3.16 as the number of H-bond acceptor groups (N, O, S, F, Cl, Br, I). None of the derivatives tested contained a halogen atom (F, Cl, Br or I). They all contained nitrogen (N) and oxygen (O) atoms and therefore variations in this parameter were due to the various groups attached to the molecule at the R_1, R_2 or R_3 positions. There was an extra nitrogen atom in lysylproline but Key Finding 6 was that lysylproline was a poor inhibitor. There were extra sulphur atoms in the methionyl and cysteinyl derivatives but Key Finding 4a was that an R_1 thiol group increased IC_{50}. There was an extra oxygen atom on serylproline but Key Finding 5 was that an R_1 hydroxyl group had no effect on IC_{50}. The other derivatives with extra oxygen atoms were the aspartyl and glutamyl derivatives. In those cases, extra carboxyl groups decreased IC_{50} but esterifying the groups increased IC_{50}. It was the structure of the groups containing the oxygen atoms rather than the extra oxygen atoms that affected IC_{50}. Taken together, these results agree with the statistical analysis that the presence of extra sulphur, nitrogen or oxygen atoms significantly increased IC_{50}.

3.3.3.3.2 Correlation of parameters for R_1, R_2, and R_3 groups with IC_{50}

Molecular parameters were also calculated for the structures in the R_1, R_2 and R_3 positions. A Pearson Correlation was then performed (using SPSS) on all the parameters (including IC_{50}) to identify correlations between parameters. The results are presented in Appendix 5. Figure 3.18 shows the correlation between IC_{50} and the parameters for R_1, R_2 and R_3. There were no significant relationships between the R_1 or R_3 parameters and IC_{50}. It can be seen that the only significant correlations are between the R_2 position and IC_{50}. The five parameters relating to the R_2 position all cross-correlated with a Pearson coefficient of 1 meaning that there was a perfect linear relationship between them. There were only two structures tested at the R_2 position, hydrogen and a benzoxy group and only for five compounds. The significant correlation between the R_2 parameters and IC_{50} means that the larger benzoxy group caused a significant increase in IC_{50}. This was seen in Key Finding 14 where the addition of a benzoxy group to the R_2 position decreased affinity for the transporter.

Table 3.18 Pearson correlation of R_1, R_2, and R_3 group parameters with IC_{50}

Parameter[1]	Correlation with IC_{50}	P value[2]
MW R_1	0.002	0.990
MW R_2	**0.311**	**0.012**
MW R_3	-0.072	-0.072
LogP R_1	-0.045	0.723
LogP R_2	**0.311**	**0.012**
LogP R_3	-0.084	0.511
MR R_1	-0.052	0.684
MR R_2	**0.311**	**0.012**
MR R_3	-0.071	0.577
MSA R_1	-0.003	0.980
MSA R_2	**0.308**	**0.013**
MSA R_3	-0.077	0.547
Dip R_1	0.209	0.105
Dip R_2	**0.313**	**0.014**
Dip R_3	0.035	0.790

[1] See Table 3.16 for abbreviations

[2] A Pearson correlation, of 16 parameters (the 15 above and IC_{50}) for all of the 64 compounds tested, was performed. Significant ($p<0.05$) correlations are shown in bold and highlighted

3.3.3.3.3 Multiple regression of all parameters with IC_{50}

All the molecular and group parameters for the 64 compounds were analysed in a stepwise multiple-regression to determine IC_{50}. The most significant model is shown in Equation 3.2 below was composed of four variables listed below:

- The molecular weight of the R_2 group (MW R_2)
- The steric energy of the molecule (Steric)
- The dipole moment of the molecule (DIP)
- The molar refractivity of the R_3 group (MR R_3)

Equation 3.2 Multiple regression of molecular parameters to predict IC_{50}

$$IC_{50} = [14.238(\pm3.128)] + [0.076(\pm0.017)*\text{MW } R_2] + [-0.172(\pm0.049)*\text{Steric}] + [-1.456(\pm0.5.25)*\text{DIP}] + [0.119(\pm0.044)*\text{MR } R_3]$$

Predicted IC_{50} will be ±3.17

Molecular weight and molar refractivity correlated with a Pearson correlation of 0.99 in Table 3.17. Therefore the molecular weight of the R_2 group (MW R_2) and the molar refractivity of the R_3 group (MR R_3) can be considered as relating to the size of the R_2 and R_3 groups. The dipole moment of the molecule was related to the presence of charged groups at the R_1 or R_3 position. In Table 3.17 the steric energy of the molecule correlated with the molecular weight, Log P, molar refractivity and molecular surface area. Equation 3.2 therefore states that IC_{50} was related to the size of the R_2 and R_3 groups, and inversely related to the size of the molecule and the charge on the R_1 and R_3 groups. However, it should also be noted that the predicted IC_{50} would have a standard deviation of ±3.17. Given that most of the calculated values have IC_{50} values between 0 and 2, Equation 3.2 is not very accurate but instead indicates important parameters.

3.4 Conclusion

3.3.1 Optimum conditions for inhibition of uptake

The optimum conditions for measuring the inhibition of Gly-Pro uptake were found to be after three minutes, at pH 6, at 37°C and with DMSO at less than 2%. Proline was also added although it had no significant effect on uptake. Other experiments showed that uptake was concentration –dependent and inhibited by both dipeptides and tripeptides, as expected. More studies are needed to confirm the activity of the dipeptide transporter on days other than day 7 to find the optimal day to perform the studies. Different plastics for the cells to grow on could also be tested to find optimal uptake.

3.3.2 The effect of dipeptide and non-dipeptide derivatives on uptake

A range of dipeptide and non-dipeptide derivatives was tested for their inhibitory activity against the uptake of Gly-Pro. The derivatives were then grouped into series leading to the identification of a number of structure-activity relationships. A statistical analysis correlating IC_{50} with molecular parameters only found significant relationships with the heat of formation and number of hydrogen-bond acceptor atoms in the molecule. A second analysis found significant links between IC_{50} and the size of the molecule, R_2 and R_3 groups and the dipole moment of the molecule. These results indicated that while molecular properties did affect IC_{50}, the size, charge and composition of the R_1, R_2 and R_3

groups caused the most significant effects. Optimising inhibitory activity should inform the optimisation of substrates for transport by the peptide transporter.

4. DEVELOPMENT OF THE CALCEIN-AM ASSAY TO INVESTIGATE INHIBITORS OF P-GLYCOPROTEIN

ABSTRACT:

Previous studies have developed a number of methods to quantify the level of p-glycoprotein activity inside cells and the inhibitory effects of compounds on the transporter, these included accumulation assays. The cells are incubated with the non-fluorescent acetoxymethyl ester of calcein that easily penetrate the cell membrane. Inside the cell they can either be pumped out by p-glycoprotein or be hydrolysed to the non-permeable fluorescent dyes. The activity of p-glycoprotein is therefore inversely proportional to the intracellular fluorescence. The fluorescence of calcein was shown to be linear with respect to concentration and quenched by the addition of cobalt chloride. There was no significant change in intracellular calcein due to the presence of mannitol (25-100 μM; $p>0.05$) but there was in the presence of cyclosporin A (50-100 μM; $p<0.01$). Incubating the cells with the test solution for 30 minutes before the addition of the ester resulted in a significant ($p<0.001$) difference between the internal standard and positive control compared to no incubation at this point. The extracellular calcein could be quenched at any time up to one hour after the thirty-minute post-calcein incubation and the resulting intracellular calcein levels would still be significantly ($p<0.01$) different from each other. Terfenadine ($p<0.05$ at 25 - 100 μM), found to be a potent inhibitor of p-glycoprotein in previously published studies, was a significant inhibitor at a lower concentration than verapamil ($p<0.05$ only at 100 μM), another known inhibitor. The normal short-term exposure to probenecid or quercetin, or the longer-term exposure to buthionine sulfoximine (75 μM for 24 hours) caused no significant ($p>0.05$) effect on calcein accumulation. Those compounds were MRP inhibitors therefore it was concluded that MRP plays a minor, non-significant role in the modified assay. Nifedipine, an inhibitor of CYP3A4 but not p-glycoprotein or MRP, did not have a significant effect on the assay. Taken together, it was concluded that the modified assay was specific for p-glycoprotein.

4.1 Introduction

4.1.1 Quantification of p-glycoprotein activity

Once p-glycoprotein had been associated with multidrug resistance, studies focused on quantifying p-glycoprotein activity to allow large-scale screening of compounds that might inhibit p-glycoprotein and reverse multidrug resistance. An early study used immunohistochemical staining of individual myeloma cells from a patient and correlated them to a number of myeloma cells lines with known p-glycoprotein activity to quantify p-glycoprotein activity in each patient [Dalton *et al.*, 1989]. This technique was technically difficult and time-consuming so later studies looked at combining fluorescent compounds with flow cytometry.

Some anthracyclines (*e.g.* daunorubicin) are used to treat cancer but are also substrates for p-glycoprotein and fluorescent. An *in vitro* uptake study by on-line flow cytometry showed that cells accumulate daunorubicin [Herweijer *et al.*, 1990]. In leukaemia cells expressing p-glycoprotein, this accumulation could be significantly increased by the addition of cyclosporin and verapamil. It was proposed that that drug resistance could be overcome by treatment with cytotoxic drugs in combination with p-glycoprotein inhibitors. However, because daunorubicin is a cytotoxic agent, other non-toxic fluorescent probes were studied to identify p-glycoprotein activity.

The intracellular distribution of rhodamine-123, daunorubicin, and doxorubicin was studied using laser scanning microscopy and confocal microscopy [Weaver *et al.*, 1991]. All three compounds were localised to both the plasma membrane and intracellular compartments. Treatment with verapamil resulted in an increase in the amount of all three compounds associated with the cells. However, after treatment with verapamil, rhodamine-123 relocalised to cellular locations that were different to the locations of daunorubicin and doxorubicin. These results showed that rhodamine-123 was an adequate non-toxic model for daunorubicin. Rhodamine 6G in human epidermal carcinoma (KB) cells [Yoshimura *et al.*, 1990] and Hoechst 33342 in small cell lung cancer, adenocarcinoma, and large cell carcinoma cells [Morgan *et al.* 1989] have both successfully been used to show the effect of other compounds on their accumulation inside cells expressing p-glycoprotein. The fluorescein derivative Fluo-3 has been shown to be a good indicator for p-glycoprotein activity in human T-cell lymphoblastic leukaemia cell

lines and was more rapid and more sensitive than the flow cytometric measurement of doxorubicin, whose fluorescence is quenched upon binding to DNA [Wall *et al.*, 1991].

P-glycoprotein is similar but different to the multidrug resistance protein (MRP). They have a different but overlapping range of substrates and inhibitors. Glutathione is needed for the transport of MRP substrates such as daunorubicin and vincristine [Versantvoort *et al.*, 1994]. Rhodamine-123 has been shown to be a substrate for both p-glycoprotein (using the mouse cell line EMT6/AR1.0) and MRP (using the human lung cancer cell line COR-L23/R) [Twentyman *et al.*, 1994]. The transport of rhodamine-123 may be influenced by both p-glycoprotein and MRP.

A different approach to quantifying p-glycoprotein activity was to use the hydrophobic acetoxymethyl (AM) ester of the dyes. The AM ester of Fura-2 and other indicators rapidly diffuses through the cell membrane. Cytoplasmic non-specific esterases rapidly hydrolyse the esters trapping the non-permeable hydrophilic free acids inside the cell. An inward concentration gradient of the AM ester leads to cellular accumulation of the free acid. This technique has been used to introduce non-toxic dyes into cells to measure intracellular ion concentrations [Tsien, 1983].

Mouse fibroblasts expressing p-glycoprotein have been shown to actively extrude the AM esters of a number of dyes including Fura-2, Quin-2, Indo-1, Fluo-3, BCECF and calcein [Homolya *et al.*, 1993]. Known substrates and inhibitors of p-glycoprotein blocked their extrusion. There is a reduced accumulation of the dyes in the fibroblasts expressing p-glycoprotein compared to untransfected cells. The accumulation was restored to normal in the presence of verapamil, a substrate for p-glycoprotein (and used as a reversible inhibitor). It was concluded that the AM esters are actively extruded by p-glycoprotein against their concentration gradient, reducing the accumulation of the free acids inside the cells.

Further studies on calcein showed that the rate of calcein accumulation in human p-glycoprotein-expressing cells was significantly lower than in control cells, while various drug-resistance reversing agents (including cyclosporin A and UIC2 monoclonal antibody) increased calcein accumulation only in the transfected cells [Hollo *et al.*, 1994]. Cell-surface p-glycoprotein expression has been combined with this calcein accumulation method to quantify p-glycoprotein expression and activity in clinical samples [Homolya *et*

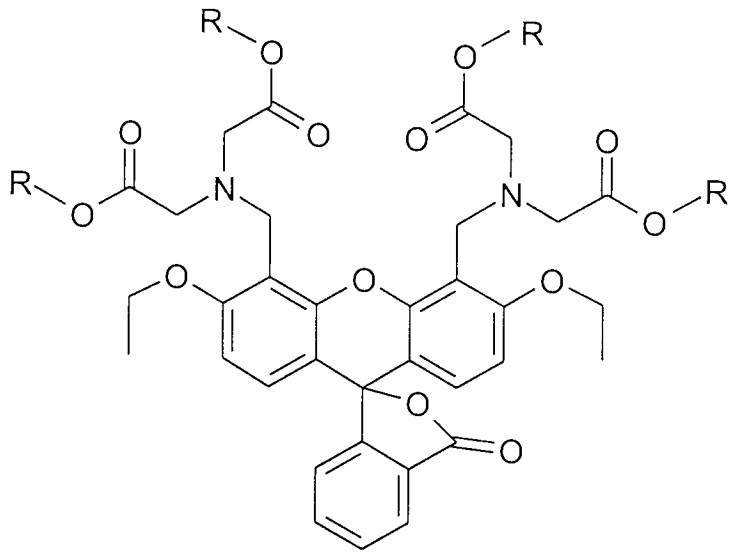
al., 1996]. This approach was reproducible and more sensitive than other assays using flow cytometry.

The fluorescence of calcein is practically pH- (see Figure 4.2 below), calcium-, and magnesium-insensitive [Wallach & Stock, 1963] and has a high intensity in the visible range. This compares favourably to the fluorescence of Fluo-3, which is very sensitive to the concentration of calcium and has a low intensity [Minta *et al.*, 1989]. Calcein AM has been shown to have cytotoxic activity against a number of cell lines [Liminga *et al.*, 1995]. The drug was less active in cells expressing p-glycoprotein although there was no direct relationship between the concentration of cytoplasmic calcein and cytotoxic efficacy. Further studies showed that solid tumours such as non-small cell lung cancer and sarcomas were most sensitive to calcein AM [Jonsson *et al.*, 1996] but that more research was needed.

A study was performed to see if calcein was transported by MRP expressing human acute myeloblastic leukaemia cells examined using flow cytometry [Feller *et al.*, 1995]. Calcein was transported by MRP in an ATP-dependent process and the transport was inhibited by probenecid and vincristine. As stated above, glutathione is needed for the transport of MRP substrates such as daunorubicin and vincristine [Versantvoort *et al.*, 1994] but was not needed for calcein transport. The structures of calcein and calcein AM are shown in Figure 4.1 below. Since calcein is a strongly negatively charged molecule, the transport of calcein by MRP supports the findings that MRP acts as a multispecific organic anion transporter (MOAT) [Muller *et al.*, 1994]. It was concluded that calcein is transported by MRP but not by p-glycoprotein and that this transport can be inhibited by compounds including probenecid.

Thirteen cell lines with different levels of p-glycoprotein and MRP expression were used to assess the calcein accumulation method [Legrand *et al.*, 1998]. There was a good correlation between p-glycoprotein expression and the effect of cyclosporin A ($p<0.0001$) and MRP expression and the effect of probenecid ($p<0.0003$). It was concluded that the calcein accumulation method could be used with modulators to measure p-glycoprotein and MRP activity.

Figure 4.1 The structure of calcein and calcein AM



Where:
R = H for calcein
R = CH_2OOCCH_3 for calcein AM

4.1.2 Calcein accumulation assays

A microtitre plate-based fluorometric assay using calcein as a probe was developed to replace the flow cytometry methods [Liminga *et al.*, 1994]. Cells from three cell lines (myeloma RPMI 8226 and two of its doxorubicin-resistant sublines, dox40 and dox6) were washed in PBS containing 5mM glucose and incubated at 37°C for 30 minutes with calcein-AM and the test compound. The plate was then centrifuged for 5 minutes at 200 *g* and then washed twice with PBS (to remove extracellular calcein and calcein-AM) before the fluorescence was read by a microtitre-plate scanning fluorometer.

The cell lines expressing p-glycoprotein showed poor calcein accumulation compared to the parent cell line. Accumulation of calcein was inversely related to p-glycoprotein expression. Cyclosporin, vincristine (both p-glycoprotein substrates) and cyanide (an ATPase inhibitor) all increased the accumulation of calcein in the p-glycoprotein expressing cell lines (in a dose-dependent manner) but not in the parent cell line.

A screening assay based on vincristine-resistant Caco-2 cells (Caco-2VCR) has also been developed [Eneroth *et al.*, 2001]. The cells were cultured as monolayers in 96-well plates. The culture medium was replaced by 50 µl HBSS (pH 7.4) containing the test compound

and incubated at 37°C for 15 minutes in a plate shaker. An additional 150 µl of test solution, including 1.33 µM calcein AM, was added to the cells and incubated for 20 minutes. After incubation, the solution was removed and 200 µl HBSS was added and the fluorescence in the cells read. The method was promoted as an improvement on previous methods as the cells were easily cultured and there was no need for a centrifugation step. P-glycoprotein was the predominant efflux system in the assay as MDR1 was overexpressed while MRP was not. Fourteen known p-glycoprotein modulators, with a wide range of structures, were tested.

4.1.3 Calcein and cobalt chloride

The fluorescence of calcein metal chelates at pH 7.4 has been known for many decades [Wallach & Steck, 1963]. Table 4.1 below (adapted from Table 1 Wallach & Steck 1963) shows the fluorescence of the metal chelates.

Table 4.1 The fluorescence of calcein (20 µM) and some of its metal chelates (200 µM) at pH 7.4 (495 nm)

Metal ion	Fluorescence (arbitrary units)
None	991
Mg^{+2}	992
Zn^{+2}	988
Al^{+3}	730
Mn^{+2}	80
Cu^{+2}	10
Ni^{+2}	4
Co^{+2}	2

The chelates of metal ions Mg^{+2} and Zn^{+2} had the same fluorescence as the free calcein; Al^{+3} and Mn^{+2} had lower fluorescence while Cu^{+2}, Ni^{+2} and Co^{+2} quenched nearly all the fluorescence. Due to the concentrations of calcein and metal ions used it was concluded that calcein forms strong one-to-one chelates with Cu^{+2}, Ni^{+2} and Co^{+2} that are not fluorescent.

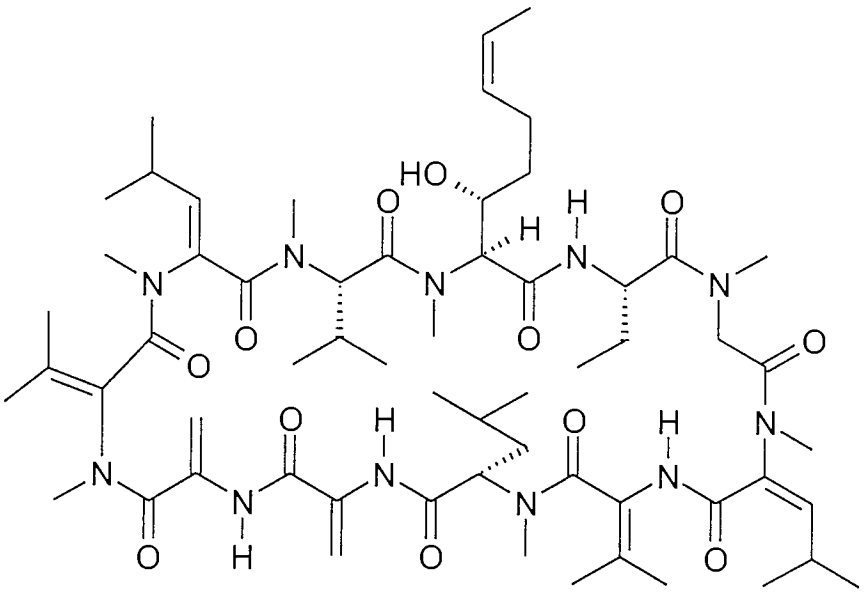
The quenching of calcein by cobalt ions has been used in conjunction with a calcein accumulation assay. Cell lines expressing p-glycoprotein or MRP were incubated with calcein AM and the calcein formation followed over time by measuring the intracellular fluorescence [Essodaigui *et al.*, 1998]. The intracellular calcein could be distinguished from the extracellular calcein by the addition to the medium of $CoCl_2$ (2μM) that completely quenched the extracellular fluorescence. This quenching step removed the need for a centrifugation step.

4.1.4 Inhibitors of p-glycoprotein

4.1.4.1 Cyclosporin

Cyclosporin is a widely used immunosuppressive agent produced by *Tolypocladium inflatum*. Structurally, it is a lipophilic cyclic polypeptide consisting of eleven amino acids and with a molecular weight of 1202.63 Da (see Figure 4.2 below).

Figure 4.2 The structure of cyclosporin A



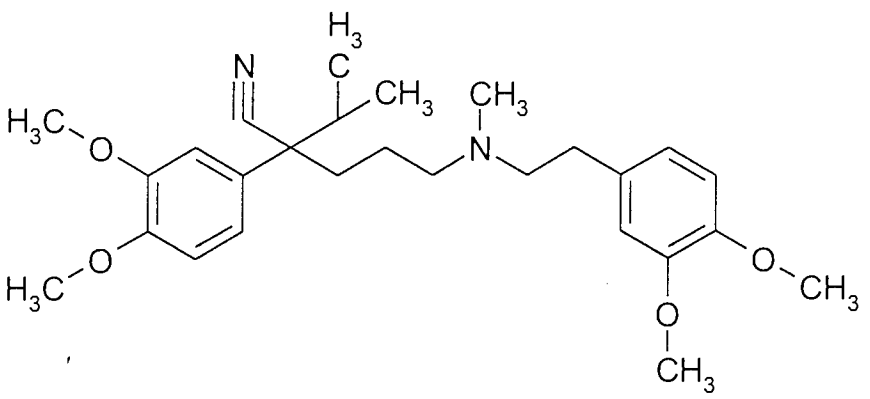
Analysis of data from a number of studies showed that, in the absence of intestinal metabolism, on average 65% of an oral dose is absorbed [Wu *et al.*, 1995], however cyclosporin has a low (14-36%) oral bioavailability [Hebert *et al.*, 1992]. It was concluded that the difference was due to clinically significant intestinal metabolism of cyclosporin by CYP3A. Cyclosporin has also been shown to be transported by p-glycoprotein. Cyclosporin competed with vinca alkaloids in multidrug resistant Chinese hamster cells for a binding site on p-glycoprotein [Tamai & Safa, 1990] and a cell line derived from porcine

kidney proximal tubule and transfected with p-glycoprotein on the apical surface was able to transport cyclosporin out of the cell [Saeki *et al.*, 1993]. A study of hepatic CYP3A4, intestinal CYP3A4 and p-glycoprotein activity in kidney transplant patients treated with cyclosporin was performed [Lown *et al.*, 1997]. It was found that 56% of the variability in cyclosporin clearance was due to variation in hepatic CYP3A4 activity, 17% was due to variations in p-glycoprotein activity and that variations in intestinal CYP3A4 activity had no effect. It was concluded that p-glycoprotein had a significant role in the first-pass elimination of cyclosporin, presumably by being a rate-limiting step in absorption. Drug interactions with cyclosporin that had previously been ascribed to intestinal CYP3A4 may instead be mediated by interactions with p-glycoprotein. In summary, cyclosporin has been shown to be a substrate of p-glycoprotein.

4.1.4.2 Verapamil

Verapamil is a calcium antagonist in a variety of cardiac tissues. Its structure is shown in Figure 4.3 below. It is used to treat patients with hypertension, coronary artery disease and stable angina pectoris [Brogden & Benfield, 1996]. It undergoes extensive metabolism by a number of cytochrome P450 isozymes [Mikus *et al.*, 1990]. Dosing humans with [^{14}C]-verapamil led to ~70% of the ^{14}C being excreted in urine within five days and only 3-4% of the dose excreted in urine was the parent drug [Eichelbaum *et al.*, 1979].

Figure 4.3 The structure of verapamil



The uptake into and cytotoxicity of vincristine in adriamycin-resistant P388 murine leukaemia cells and the uptake into and cytotoxicity of adriamycin in vincristine-resistant P388 murine leukaemia cells were both enhanced by the co-administration of verapamil [Tsuruo *et al.*, 1982]. It was concluded that there is a common transport mechanism for

vincristine and adriamycin that is responsive to verapamil. Inhibition studies have shown that verapamil and some, but not all, of its CYP metabolites are potent inhibitors of p-glycoprotein-mediated transport of digoxin in Caco-2 cells [Pauli-Magnus *et al.*, 2000]. A calcein accumulation assay using Caco-2 cells expressing p-glycoprotein but not MRP showed that verapamil inhibited p-glycoprotein (EC_{50} 70 μM) but not as strongly as cyclosporin A (EC_{50} 7 μM) [Eneroth *et al.*, 2001]. It was concluded that verapamil and its CYP metabolites are substrates for p-glycoprotein.

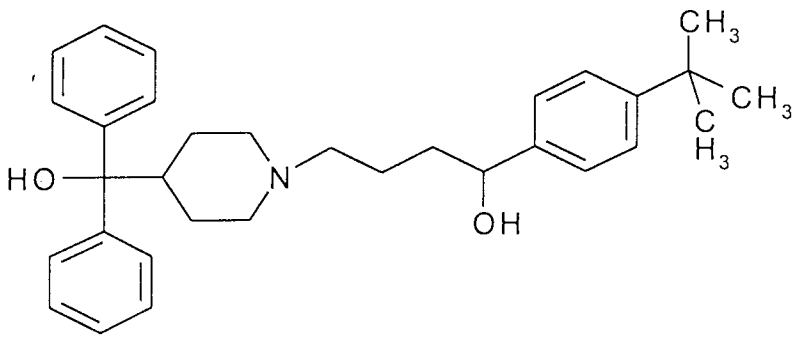
The effect of verapamil on the transport of leukotriene C_4 through MRP1-enriched membrane vesicles was studied to clarify the effect of verapamil on MRP [Loe *et al.*, 2000]. It was found that verapamil is a poor direct inhibitor of MRP1-mediated transport but strongly stimulates glutathione transport by this protein, which may itself reduce MRP activity. It was concluded that verapamil may be seen to inhibit transport in MRP-expressing cells, however the magnitude of this effect will vary between different cell lines as glutathione levels vary.

In summary, verapamil is a substrate for a number of cytochrome P450 isozymes including CYP3A4 and an inhibitor of p-glycoprotein. However, it has an indirect effect on MRP activity that may have a significant effect on the assay if the cells have high levels of glutathione.

4.1.4.3 Terfenadine

Terfenadine is a non-sedating antihistamine and a substrate for p-glycoprotein (see Figure 4.4 below for its structure).

Figure 4.4 The structure of Terfenadine



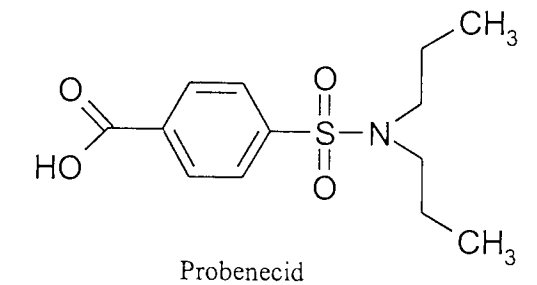
It has an oral bioavailability of less than 1% due to extensive first-pass metabolism [Garteiz *et al.*, 1982]. The major metabolite in humans is terfenadine carboxylate (also an antihistamine), excreted *via* the urine and faeces. Increased plasma levels of terfenadine are cardiotoxic leading to convulsions [Davies *et al.*, 1989] and i*n vitro* studies showed that terfenadine was as potent as quinidine as a blocker of the potassium current in isolated feline myocytes [Woosley *et al.*, 1993]. Terfenadine metabolites had no effect. It was concluded that cardiotoxic effects are the result of the parent drug and factors that impair the normally rapid metabolism of terfenadine. The cardiotoxicity led to the voluntary discontinuing of all terfenadine-containing products in the United States.

Terfenadine is metabolised by CYP3A4 into hydroxyterfenadine and azacyclonol [Raeissi *et al.*, 1999]. Both metabolites were found to be substrates for p-glycoprotein. A 40-year old breast cancer patient given doxorubicin and terfenadine developed neutropenia (decrease in the number of neutrophils) [Hait *et al.*, 1992]. It was hypothesised that terfenadine increased the sensitivity of cells to doxorubicin leading to toxicity. However, when the patient stopped taking terfenadine the neutropenia continued [Hait *et al.*, 1993]. Although the terfenadine was not the cause of the symptom, research continued on its relationship with doxorubicin and p-glycoprotein. Terfenadine was shown to sensitise MCF-7/ADR human breast cancer cells and L1210/VMDRC.06 murine leukaemia cells to doxorubicin, vincristine, colchicines and etoposide [Hait *et al.*, 1993]. It increased the accumulation of doxorubicin and rhodamine-123 and it was concluded that method of action of terfenadine is through the inhibition of p-glycoprotein. Further studies showed that terfenadine is transported at a faster rate from basolateral-to-apical rather than apical-to basolateral and that it inhibited the transport of digoxin through Caco-2 cells [Kim *et al.*, 1999]. It was concluded that terfenadine is an inhibitor of p-glycoprotein. In summary, terfenadine is a CYP3A4 substrate and a p-glycoprotein inhibitor.

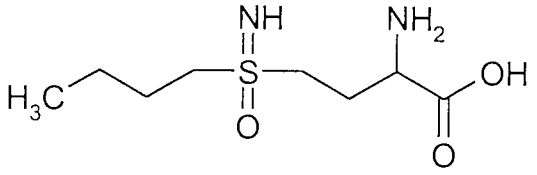
4.1.5 Inhibitors of MRP

Three inhibitors of MRP were studied: probenecid, buthionine sulfoximine and quercetin. The structures of the three compounds are shown in Figure 4.5 below.

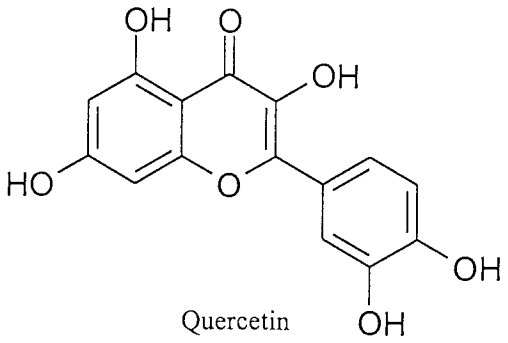
Figure 4.5 The structures of probenecid, buthionine sulfoximine and quercetin



Probenecid



Buthionine Sulfoximine



Quercetin

4.1.5.1 Probenecid

Probenecid is used to treat gout [Burgos & Capone, 1996] but has also been shown to increase the serum concentrations of a number of drugs. Probenecid significantly increased serum concentrations of amoxycillin (above the minimum bactericidal concentration for this antibiotic) in humans for up to 18 hours after a single 3g oral dose [Shanson *et al.*, 1984] and increased serum concentrations of cidofovir (an antiviral nucleotide analogue) in rabbits by 65% after only 15 minutes [Cundy *et al.*, 1996]. These studies show that probenecid inhibits the transport of organic anions.

The inhibitory effect on daunorubicin or vincristine of probenecid in cell lines that expressed MRP but not p-glycoprotein (HL60/AR human myeloid leukaemia and H69/AR human small-cell lung carcinoma cells) and in cell lines that over-expressed p-glycoprotein (HL60/TOX human myeloid leukaemia and P388/ADR murine leukaemia cells) was

studied [Gollapudi *et al.*, 1997]. Probenecid reversed resistance in the MRP-expressing cells but not the p-glycoprotein-expressing cells. It was concluded that probenecid inhibits MRP but not p-glycoprotein.

The efflux of calcein out of non-small lung cancer cells (SW-1573) transfected with the MRP gene has been shown to be ATP-dependent and inhibited by probenecid [Feller *et al.*, 1995]. The expression of MRP in thirteen cell lines was measured using MRPm6 antibodies [Legrand *et al.*, 1998]. There was a significant correlation ($r=0.91$, $p=0.0003$) between MRP expression and the size of the effect of probenecid on calcein efflux (measured by flow cytometry). There was no correlation between MRP expression and the effects of probenecid on rhodamine-123 and daunorubicin efflux and uptake or between p-glycoprotein expression and the effect of probenecid on the three probes used. It was concluded that calcein-AM can be used to determine the presence of both MRP (effect of probenecid) and p-glycoprotein (effect of cyclosporin A) activity.

Probenecid has been used as an MRP inhibitor to prove that a calcein-AM extrusion assay was specific for p-glycoprotein [Eneroth *et al.*, 2001]. The presence of probenecid (5 mM) resulted in a marginal increase (1.8x control as opposed to 9.3x control for cyclosporin A) in calcein retention in Caco-2VCR25 cells (vincristine resistant Caco-2 cells). Indometacin (another MRP inhibitor) caused a linear increase in calcein retention between 0.25 mM and 1 mM (1.5x control to 3.8x control). It was concluded that MRP played a minor role in that assay. In summary, probenecid has been used as an MRP inhibitor in many systems.

4.1.5.2 Buthionine Sulfoximine

Glutathione (L-γ-glutamyl-L-cysteinylglycine) is synthesised intracellularly by the consecutive actions of γ-glutamylcysteine synthetase and glutathione synthetase. Buthionine sulfoximine inhibits γ-glutamylcysteine synthetase and injection of the chemical into mice decreased the level of glutathione in the kidney to <20% of the level in control mice [Griffith & Meister, 1979].

In early studies of multidrug resistance not mediated by p-glycoprotein, buthionine sulfoximine was found to increase daunorubicin toxicity in several cell lines [*e.g.* Lutzky *et al.*, 1989] and also doxorubicin toxicity in several cell lines [*e.g.* Meijer *et al.*, 1991]. As

the levels of glutathione and glutathione-S-transferase were increased in the MDR GLC4/ADR cells compared to the parental cells [Meijer *et al.*, 1991], it was suggested that detoxification of doxorubicin by glutathione was an important factor contributing to the doxorubicin resistance. A later study on three MRP-expressing MDR human lung tumour cell lines found that buthionine sulfoximine reduced cellular glutathione levels by 60-80% and reduced resistance to daunorubicin, vincristine and rhodamine-123 [Versantvoort *et al.*, 1995]. Treatment with buthionine sulfoximine (25 μM for 20 hours) led to an accumulation of daunorubicin in small-cell lung cancer cells expressing MRP but not in small-cell lung cancer cells expressing p-glycoprotein [Versantvoort *et al.*, 1995]. It was concluded that drug transport in MRP-expressing, but not p-glycoprotein-expressing, cell lines can be regulated by intracellular glutathione levels, specifically by treatment with buthionine sulfoximine. As noted in the previous chapter, a study of the efflux of calcein by MRP (measured by flow cytometry) in non-small lung cancer cells found that glutathione depletion (after incubation for 20 hours with 25 μM buthionine sulfoximine) had no effect [Feller *et al.*, 1995]. It can be predicted that glutathione-depletion by buthionine sulfoximine should have no effect on the assay.

4.1.5.3 Quercetin

Quercetin is one of a group of over 4000 naturally available plant phenolics whose isolation and biological identification were first described by Szent-Gyorgyi in the 1930s. The edible portions of some foodstuffs have an unusually high concentration of quercetin including onions (284-486 mg/kg), kale (110 mg/kg), French beans (32-45 mg/kg), broccoli (30 mg/kg), lettuce (14 mg/kg) and tomatoes (8 mg/kg) [Hertog *et al.*, 1992]. The quercetin content of a number of drinks has also been studied [Hertog *et al.*, 1993]. Beer, coffee, chocolate milk and white wine all contained approximately 1 mg/L quercetin. Red wines contained 4-16 mg/L, tomato juice contained 13 mg/L and tea infusions contained 10-25 mg/L. Other fruit juices contained approximately 5 mg/L. Quercetin is unavoidably consumed daily. It has been shown to have anticancer, antiviral and beneficial cardiovascular effects [Formica & Regelson, 1995].

As stated previously, two methods to measure p-glycoprotein activity are the accumulation of radiolabelled daunorubicin and the accumulation of fluorescent rhodamine-123, although both will also measure MRP activity. The effect of a number of flavonoids including quercetin on both these assays using human cancer cell lines overexpressing

either MRP or p-glycoprotein was studied [Versantvoort *et al.*, 1996]. Quercetin increased daunorubicin accumulation in the MRP cell lines after a 60-minute incubation but not the p-glycoprotein cell lines. However, quercetin (and the other flavonoids) reduced the rhodamine-123 accumulation in the MRP cell lines after a 60-minute incubation. Other modulators of MRP activity (such as verapamil and buthionine sulphoximine) increased rhodamine-123 accumulation except for sodium azide, which reduced it. Rhodamine-123 accumulation depends on the mitochondrial membrane potential and the concentration of sodium azide used disrupts this potential without affecting cellular ATP levels. Measuring the membrane potential, using the fluorescent probe $DiOC_5$, it was found that genistein depolarised the membrane potential in both MRP-expressing and parental cells by approximately 40%. It was concluded that the mechanisms by which flavonoids interact with drug transporters is complex. Flavonoids not only affect p-glycoprotein and MRP differently but also have opposite effects on daunorubicin and rhodamine-123 accumulation assays in MRP-expressing cells.

P-glycoprotein expressed on brain capillary endothelial cells is a key component of the blood-brain barrier. Cultured mouse brain capillary endothelial cells (MBEC4 cells) have been used to study the effect of quercetin on vincristine transport [Mitsunaga *et al.*, 2000]. A concentration-dependent biphasic effect was noted. The efflux of vincristine was activated by 10 μM quercetin but inhibited by 50 μM quercetin. The lower concentration of quercetin was found to increase the phosphorylation of p-glycoprotein while the higher concentration did not. It was concluded that quercetin indirectly activates p-glycoprotein at low concentrations but directly inhibits it at high concentrations. However, as vincristine is a substrate for both p-glycoprotein and MRP it cannot be assumed that it is p-glycoprotein that is being inhibited.

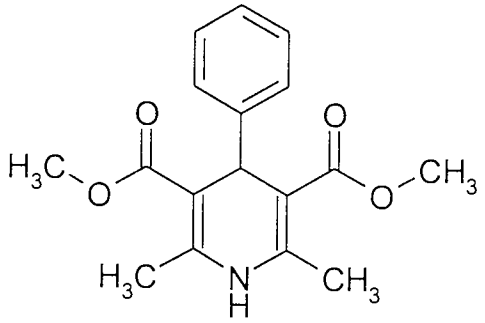
In summary, quercetin has been found to inhibit MRP activity at high concentrations but to have no effect on p-glycoprotein at high concentrations.

4.1.6 Compounds that inhibit neither p-glycoprotein or MRP

4.1.6.1 Nifedipine

The structure of nifedipine, a dihydropyridine, is shown in Figure 4.6 below.

Figure 4.6 The structure of nifedipine



Nifedipine has been known for some time to be oxidised by rat and human liver microsomal cytochrome P450 [Guengerich *et al.*, 1986] and specifically by CYP3A4 [Wacher *et al.*, 1995]. There have been a few studies of the effect of nifedipine on p-glycoprotein activity. Nifedipine had no effect on restoring doxorubicin sensitivity to resistant P388 mouse leukaemia cells [Ramu *et al.*, 1984]. There was no difference in the apical-to-basolateral and basolateral-to apical transport of nifedipine across porcine kidney cells expressing p-glycoprotein or not expressing p-glycoprotein [Kim *et al.*, 1999]. It also had no effect on the transport of daunorubicin or digoxin in p-glycoprotein-expressing porcine kidney cells [Katoh *et al.*, 2001]. However, it was shown to abolish the secretion of digoxin in Caco-2 cells [Cavet *et al.*, 1996] by reducing basolateral-to-apical transport. Inhibitors of p-glycoprotein such as verapamil and vinblastine had a similar effect. In summary, a majority of studies have shown that the CYP3A4 substrate nifedipine is not a substrate for p-glycoprotein.

4.1.7 Proposed assay

In the original microtitre plate-based fluorometric assay using calcein [Liminga *et al.*, 1994], the cells were suspended in PBS containing 5 mM glucose (160μl) and incubated at 37°C for 30 minutes with calcein-AM (20 μl) and the test compound (20 μl). The plate was then centrifuged for 5 minutes at 200 *g* and then washed twice with PBS (to remove extracellular calcein and calcein-AM) before the fluorescence was read by a microtitre-plate scanning fluorometer.

Two changes to this assay are proposed. The centrifugation step is to remove the extracellular calcein so the measured fluorescence is from the intracellular calcein. However, this requires the use of a plate centrifuge and the removal of solutions. This step

could be replaced by a simpler step through the addition of cobalt chloride that would quench the extracellular calcein. The second proposed change would be the addition of a step where the cells are incubated with the test compound before the addition of calcein, as this would give the test compound time to act.

4.1.8 Aim of chapter

The aim of this chapter was to develop a calcein-AM assay and apply the assay to compounds with known effects on p-glycoprotein and MRP to confirm its specificity through:

- Confirming the quenching effect of cobalt chloride on calcein
- Identifying the effect of incubating the cells with a test compound before the addition of Calcein-AM
- Modifying the calcein accumulation assay taking the two previous points into account
- Identifying the effect on the assay of inhibitors of p-glycoprotein
- Identifying the effect on the assay of inhibitors of MRP
- Identifying the effect on the assay of compounds which inhibit neither transporter
- Thereby identifying the specificity of the assay
- Comparing the modified assay with another p-glycoprotein assay

4.2 Materials and methods

4.2.1 Effect of pH on calcein

Solutions with a range of pH values (3-12) were aliquoted into a 96-well plate (180 μl per well) and calcein was added (1 mM, 20 μl). The fluorescence was then read.

4.2.2 Effect of cobalt on calcein in solution

Calcein (1 μM, 20 μl) was added to a range of cobalt chloride concentrations (2 μM-20 mM, 180 μl) in a 96-well plate and their fluorescence read.

4.2.3 Calcein fluorescence calibration curve

Solutions with a range of calcein concentrations (0.01 μM to 0.1 μM) were put into a 96-well plate and their fluorescence read.

4.2.4 Composition of the solutions tested

All compounds were supplied from Sigma except for Calcein-AM, which was supplied from Calbiochem.

Calcein-AM (1 mg) was dissolved in 1.005 ml DMSO to make a 1 mM solution. Aliquots (30 μl) were diluted with PBS containing 5 mM glucose (2970 μl) to make a 10 μM stock solution for the assay (final DMSO concentration of 1%).

Cyclosporin (10 mg) was dissolved in 8.3 ml of a mixture of ethanol and PBS containing 5 mM glucose (1:1) and divided into eight 1 ml 1 mM aliquots (containing 50% ethanol). The stock solution was diluted to a maximum concentration of 100 μM (which contained 5% ethanol).

Verapamil, buthione sulfoximine, probenecid, quercetin and nifedipine were separately dissolved in PBS containing 5% glucose to make 1 mM stock solutions. Terfenadine was dissolved in PBS containing 5% glucose and 10% ethanol before being diluted to make the stock solution. The 1 mM solution of terfenadine contained 3.85% ethanol. The maximum concentration of terfenadine that the cells were exposed to (100 μM) contained 0.385% ethanol.

4.2.5 Effect of pre-ester incubation duration

The protocol was the same as in Section 4.2.7 although the pre-ester incubation varied between zero and forty-five minutes.

4.2.6 Fluorescence post quenching

The protocol was the same as in Section 4.2.7 although the fluorescence was read immediately and then every fifteen minutes for one hour.

4.2.7 Effect of compounds on assay

Caco-2 cells were split, counted and resuspended in PBS containing 5 mM glucose to a concentration of $3.9x10^5$ cells/ml. The cell suspension (160µl, 624,000 cells/well) was aliquoted into a 96-well plate and the test solution added (20 µl). The plate was then incubated at 37 °C for 30 minutes (pre-ester incubation). The calcein-AM was then added (20 µl, 10 µM) and the plate incubated at 37 °C for 30 minutes (post-ester incubation). Cobalt chloride (20 µl, 20 mM) was then added to each well and the fluorescence read immediately (excitation at 485 nm, emission at 538 nm). There were three combinations of cells, calcein-AM, cobalt and PBS containing 5 mM glucose for each test compound. They were Cells, Calcein-AM & Cobalt, Cells, No calcein-AM & No Cobalt and No cells, Calcein-AM & Cobalt. The 96-well plate was set up as in Figure 4.7 below allowing the measurement of the effect of six test solutions, the positive control (100 µM cyclosporin A) and the internal standard (PBS containing 5 mM glucose), with four wells for each condition.

Figure 4.7 Layout of 96-well plate

	Cells, Calcein-AM & Cobalt	Cells, No calcein-AM & No cobalt	No cells, Calcein-AM & Cobalt
Internal standard	O O O O	O O O O	O O O O
Positive control	O O O O	O O O O	O O O O
Test solution 1	O O O O	O O O O	O O O O
Test solution 2	O O O O	O O O O	O O O O
Test solution 3	O O O O	O O O O	O O O O
Test solution 4	O O O O	O O O O	O O O O
Test solution 5	O O O O	O O O O	O O O O
Test solution 6	O O O O	O O O O	O O O O

The intracellular calcein for each solution was measured by subtracting the fluorescence of the last two combinations from the first. The intracellular calcein relative to internal standard was calculated by dividing the intracellular calcein for the test solution by the

intracellular calcein for the internal standard solution. An example calculation is shown in Section 4.3.1 below.

4.2.8 Effect of compounds on vincristine transport

Caco-2 cells were seeded on 6-well polyester inserts (diameter 24 mm, pore size 3 μm) at a density of 40,000 cells/insert. Culture media was replaced on alternate days and the cells used 21 days post-seeding. The existing culture media was aspirated and replaced with transport media (pH 7.4) (1.5 ml apical, 2.6 ml basolateral) for 15 minutes. For apical-to-basolateral (A-B) transport, transport media (pH 7.4) was added to the wells of another 6-well plate and the inserts, filled with transport media (pH 7.4) containing 10 nM [^{3}H]-vincristine sulphate and 1 μM [^{14}C]-mannitol, were immediately laid down in the wells to initiate transport at 37°C. Every ten minutes for one hour, the plate containing the inserts was removed from the incubator and placed on a rotating platform to encourage a uniform distribution of vincristine in the receiver chamber. A sample volume of 200 μl was removed from the basolateral chamber and immediately replaced with an equal volume of transport media (pH 7.4). The cells on the inserts were washed with PBS-azide (0.05% w/v) (2 x 2 ml in both apical and basolateral chambers) and collected separately. The cells were then solubilised by adding Triton-X 100 (1% v/v) (2 ml/insert), incubated overnight at 37°C and the solubilised cells collected. The cell-associated radioactivity of the donor solution, collected samples, cell washes, and solubilised cells were determined by liquid scintillation counting. Cumulative transport was calculated using Equation 4.1 below.

Equation 4.1 Determining cumulative transport

$$\text{Cumulative Amount} = (C_n * V_r) + (\sum_{n-1}^{1} C * V_s)$$

Where:

Cumulative Amount = pg

C_n Sample concentration (pg/μl) at time point n

V_r Volume of receiver chamber (μl)

$\sum_{n-1}^{1} C$ Sum of sample concentrations (pg/μl) to time point n-1

V_s Volume of sample (μl)

Cumulative flux was then calculated using Equation 4.2 below.

Equation 4.2 Determining % flux

$$\% \text{ Flux} = \frac{\text{Cumulative Amount}}{C_d * V_d} * 100\%$$

Where:

Cumulative amount = pg

C_d Concentration of donor solution (pg/μl)

V_d Volume of donor solution (μl)

Cumulative transport was calculated by correcting the cumulative amount for the amount of protein present (this allowed comparison with other published transport studies). A plot of cumulative transport (pg/mg protein) against time for each insert was used to calculate the drug permeation rate (pg/mg protein/minute). The apparent permeability coefficients of vincristine sulphate (P_{app} values) were calculated using Equation 4.3 below.

Equation 4.3 Calculating P_{app}

$$Papp = \frac{(dQ/dt)}{(A * C_0)}$$

Where:

P_{app} Apparent permeability coefficient (cm/mg protein/sec)

dQ/dt The drug permeation rate (pg/mg protein/sec)

A The nominal surface area of the cell monolayers (4.76 cm^2)

C_0 The initial drug concentration in the donor solution (pg/cm^3)

For basolateral-to-apical (B-A) transport, the same procedure was applied except that the radioactive donor solution was added to the wells and the inserts only contained transport media (pH 7.4).

The same procedure was followed when vincristine was transported in the presence of a potential inhibitor. The inhibitor (100 μM) was dissolved in transport media (pH 7.4) and the solution used to make the donor solution and top up the chambers during the experiment. A one-way analysis of variance (ANOVA with Dunnett's post-test, Instat 3) was used to determine any significant difference between the Papp values for vincristine in the presence and absence of an inhibitor.

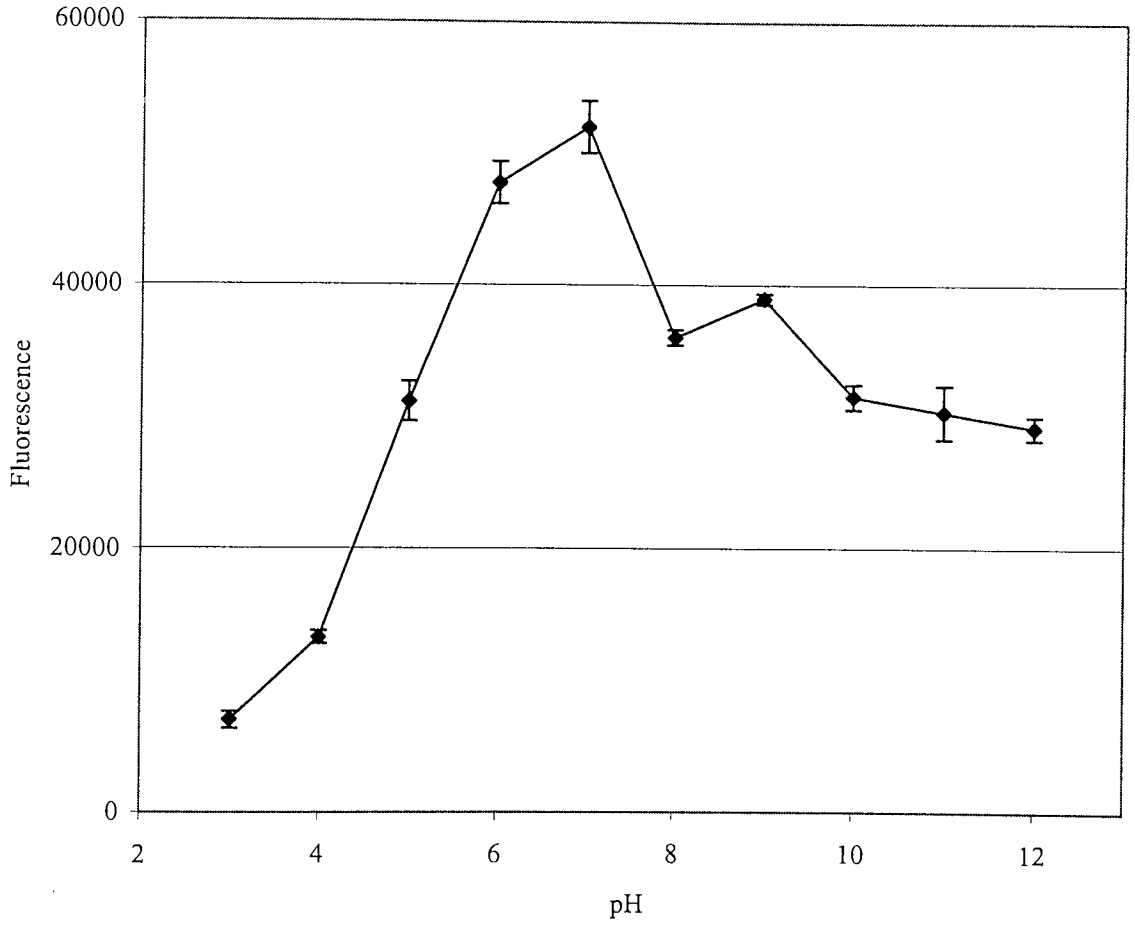
4.3 Results and discussion

4.3.1 Determining optimum conditions

4.3.1.1 Effect of pH on calcein fluorescence

The fluorescence of calcein at a range of pH values was examined (Figure 4.4 below). A one-way analysis of variance (ANOVA) of the data presented in Figure 4.8 had a P value of <0.0001. This means that the variation among the column means was significantly greater than expected by chance *i.e.* there is a relationship between pH and fluorescence.

Figure 4.8 Effect of pH on mean calcein fluorescence (n=4)



Data are mean and standard deviation of results from four wells

A Dunnett's multiple-comparison test showed that compared to the fluorescence at pH 7, the fluorescence values at all other pH values were significantly ($p<0.01$) lower. The optimum pH was therefore found to be pH 7.

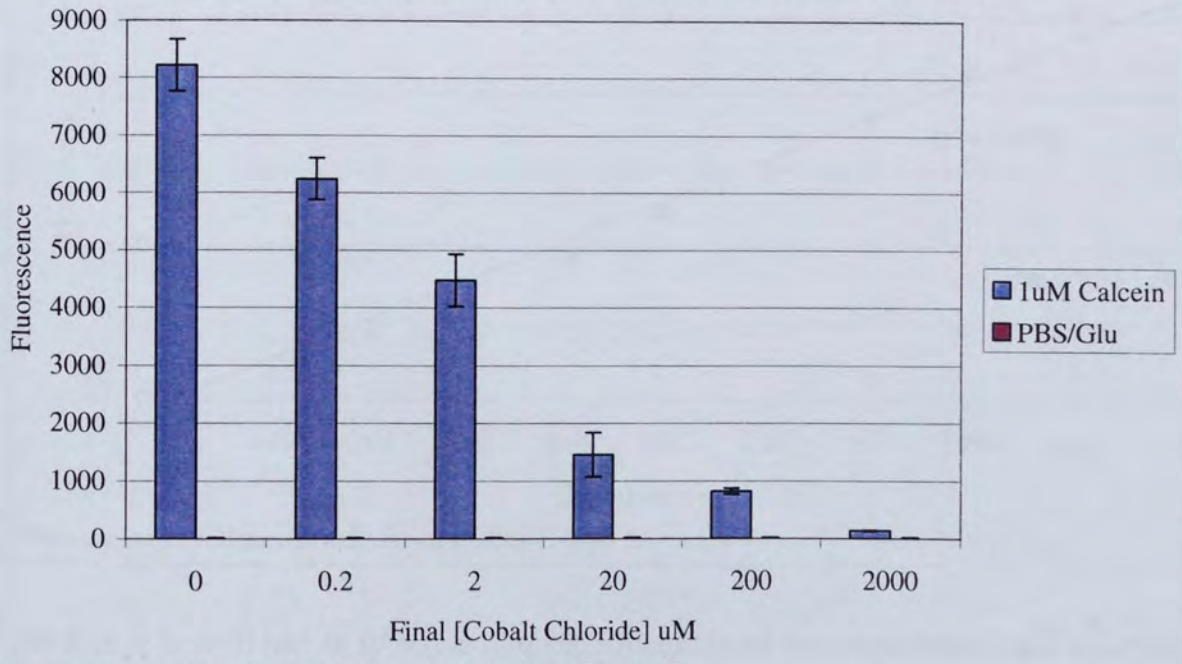
4.3.1.2 Effect of cobalt chloride on calcein fluorescence

A range of cobalt chloride concentrations (20 µl, 0-2000 µM) were added to solutions (180 µl) of 1µM calcein or PBS containing 5mM glucose and their fluorescence read (Table 4.2 and Figure 4.9 below).

Table 4.2 Effect of cobalt on fluorescence

	Mean % Control Fluorescence (± SD)	
[Cobalt] µM	1 µM Calcein	PBS containing 5 mM glucose
0 (Control)	100.0 (± 4.5)	100.0 (± 38.0)
0.2	74.9 (± 4.4)	104.9 (± 24.1)
2	54.5 (± 4.5)	62.4 (± 11.0)
20	17.7 (± 4.7)	48.3 (± 6.0)
200	10.0 (± 0.7)	124.0 (± 19.4)
2000	1.7 (± 0.1)	43.3 (± 8.4)

Figure 4.9 Effect of cobalt chloride on fluorescence of calcein



Data are mean and standard deviation of results from four wells

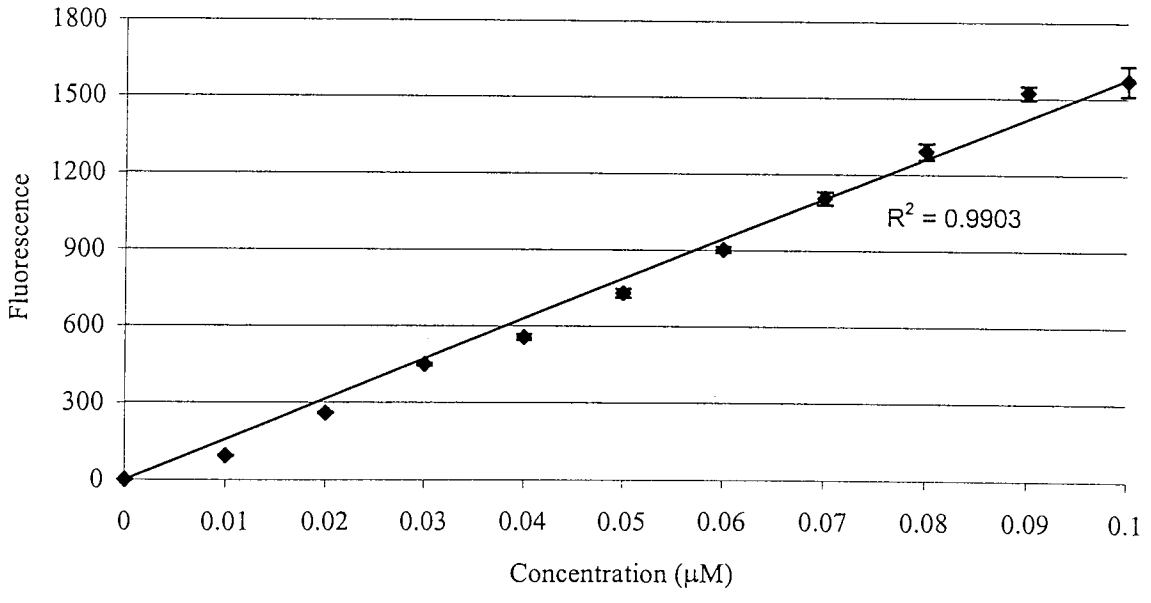
A one-way analysis of variance (ANOVA) of both the calcein and PBS containing glucose data had a P value of <0.0001. The variation between both sets of column means was significantly greater than expected by chance. This means that there was a difference in the results of the fluorescence of calcein and PBS containing glucose over the range of cobalt concentrations.

There was a clear dose-dependent reduction in calcein fluorescence as the cobalt chloride concentration increased. The addition of 2 mM cobalt chloride quenched 99% of the original calcein fluorescence. Cobalt has a different effect on the fluorescence of PBS containing glucose. Only 2 mM cobalt chloride significantly ($p<0.01$) reduces fluorescence. However, the change in fluorescence from control to 2 mM cobalt chloride (from 1.7 to 0.7), is insignificant compared to the equivalent change for calcein (fluorescence goes from 8225 to 140). It was concluded that the addition of 20 µl of 2 mM cobalt chloride would quench the extracellular calcein in the assay.

4.3.1.3 The fluorescence of calcein over a range of concentrations

The fluorescence of calcein solutions of a range of concentrations (0-0.1 µM) was measured as shown in Figure 4.10 below.

Figure 4.10 Calibration Curve for the fluorescence of calcein



Data are mean and standard deviation of results from four wells

The line of best fit had an R^2 value of 0.9903 (considered very significant) and an equation of y=15787x. This meant that the concentration of calcein could be calculated using Equation 4.4 below.

Equation 4.4 Calculating the concentration of calcein

$$Concentration = \frac{Fluorescence}{15787}$$

Where:

Concentration = μM calcein

Fluorescence = arbitrary units

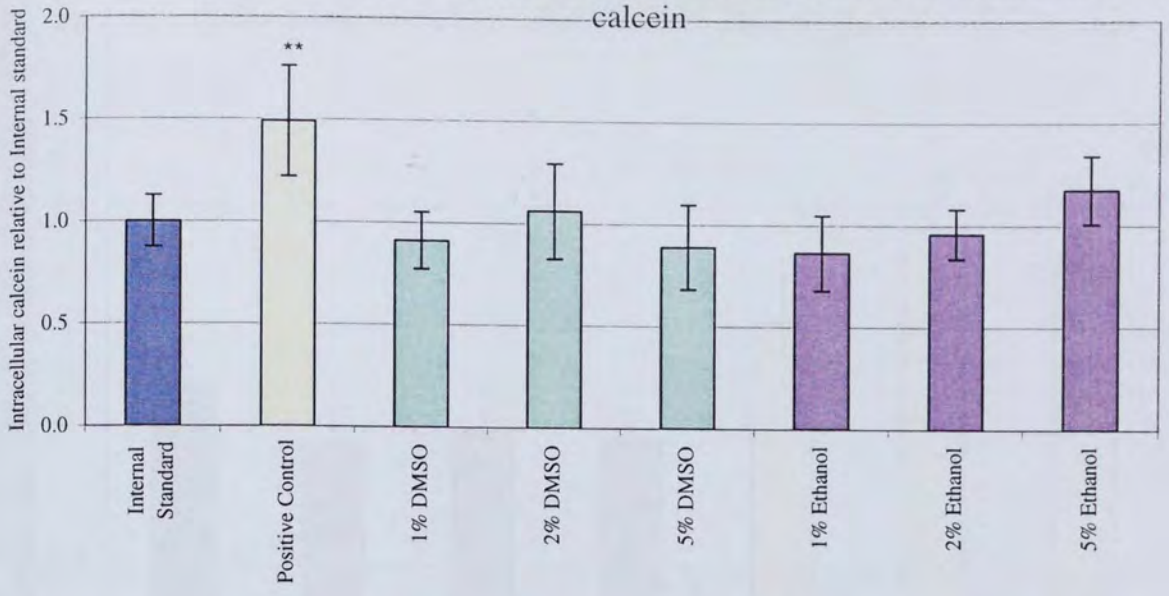
This calibration curve could be used in later experiments to measure the production of intracellular calcein during the assay. It also showed that there was a linear relationship between the concentration of calcein and fluorescence at this wavelength over a range of concentrations.

4.3.1.4 The effect of solvents on the assay

Figure 4.11 below shows the effect of the internal standard (PBS containing 5 mM glucose), a positive control (100 μM cyclosporin A) and various concentrations of two solvents, dimethyl sulphoxide (DMSO) and ethanol, on the assay.

An ANOVA analysis had a P value of 0.0016 meaning that the variation in results seen on the graph was more than expected by chance. The variations seen in intracellular calcein were due to the various conditions and not just experimental error. Only the positive control was significantly different to the internal standard. It was concluded that DMSO and ethanol could be used as co-solvents up to a concentration of 5% without significantly affecting the intracellular calcein.

Figure 4.11 Effect of dimethyl sulphoxide and ethanol on intracellular calcein



Data are mean and standard deviation of results expressed as intracellular calcein relative to the internal standard. ** Denotes a very significant ($p<0.01$) difference from the internal standard.

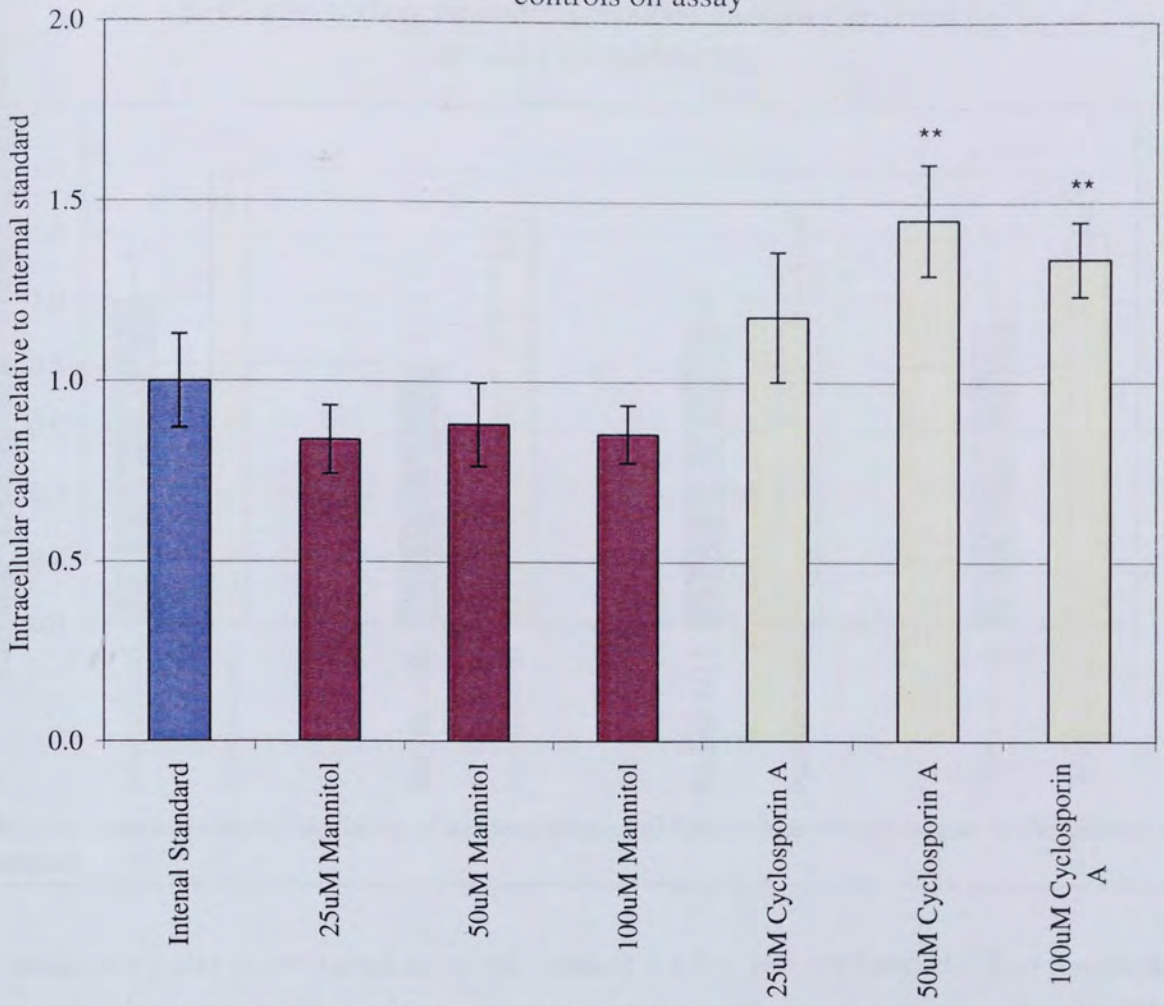
4.3.2 Assay controls

4.3.2.1 The effect of positive and negative controls

Figure 4.12 below shows the effect of the internal standard (PBS containing 5mM glucose), a negative control (100 μM mannitol as mannitol is not a substrate for p-glycoprotein or MRP) and a positive control (100 μM cyclosporin A) on the assay.

An ANOVA analysis had a P value of <0.0001 meaning that the variation among column means is greater than expected by chance and therefore variation in intracellular calcein was due to the concentration of the controls. All three concentrations of mannitol were not significantly ($p>0.05$) different to the internal standard. It was therefore concluded that the internal standard acts as a negative control. The lowest concentration of cyclosporin A (25 μM) was not significantly ($p>0.05$) different to the internal standard although both higher concentrations (50 μM and 100 μM) were significantly ($p<0.01$) greater. This was not a clear dose-dependent increase. It was therefore concluded that 100 μM cyclosporin A could be used as a positive control.

Figure 4.12 Effect of negative (mannitol) and postive (Cyclosporin A) controls on assay

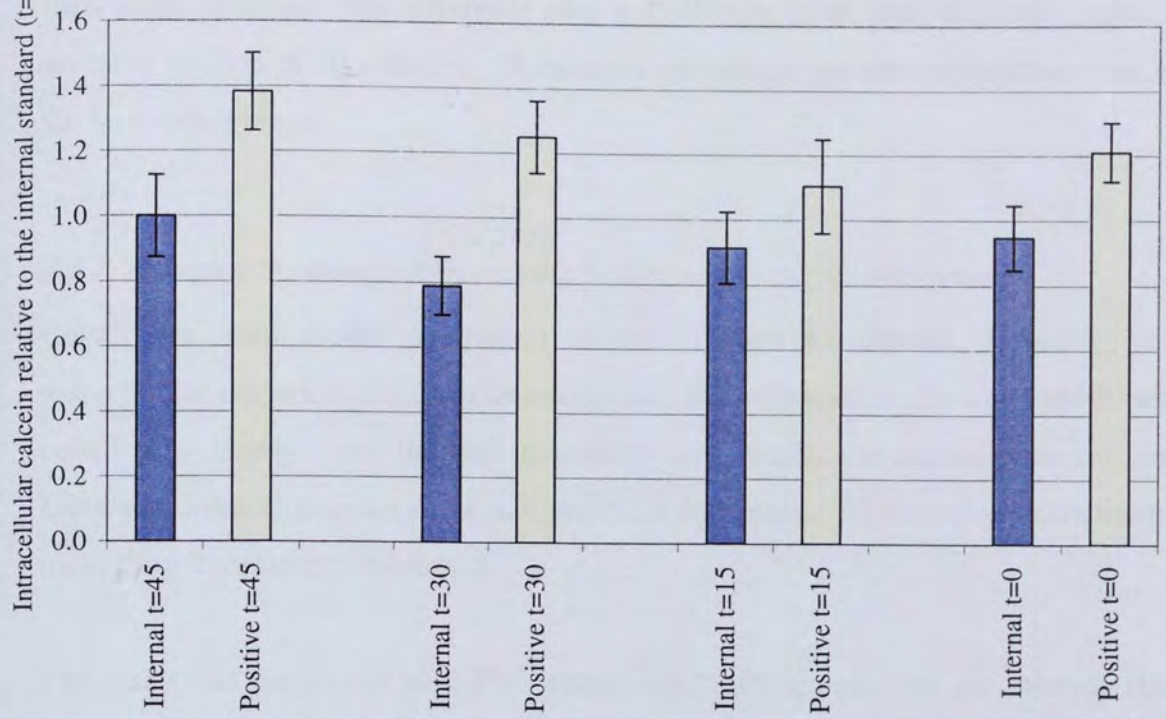


Data are mean and standard deviation of results expressed as intracellular calcein relative to the internal standard. ** Denotes a very significant ($p<0.01$) difference from the internal standard.

4.3.2.2 Varying the duration of the pre-ester incubation

The pre-ester incubation is where the cells are incubated with any test substance (*e.g.* cyclosporin A) so the compounds can affect the cells before the addition of the calcein ester. The effect of the duration of the pre-ester incubation was examined by comparing cyclosporin A against the internal standard, for different incubation durations. Figure 4.13 below shows the intracellular calcein relative the internal control with a 45 minute pre-ester incubation.

Figure 4.13 The effect of the duration of the pre-ester incubation (0-45 minutes) on the internal standard and positive control (100 μM Cyclosporin A)



Data are mean and standard deviation of results expressed as intracellular calcein relative to the internal standard.

At each time-point a two-tailed unpaired Student's t-test was performed. The results are shown in Table 4.3 below.

Table 4.3 Significance of the difference between the internal standard and positive control at different pre-ester incubation durations

Duration	P value	Significance
45	<0.01	**
30	<0.001	***
15	>0.05	ns
0	<0.01	**

Where significant values are highlighted in blue and ns = not significant

There was a significant difference when there was no pre-ester incubation. The cyclosporin A will inhibit the p-glycoprotein during the post-ester incubation as well as the pre-ester incubation therefore it was unsurprising that there was a difference at this time-point. Although there was a difference between the positive control and internal standard

after a 15-minute pre-ester incubation, this difference was not significant. This was probably due to the slightly larger standard deviations at this time-point. It can be seen that the most significant difference occurred when there was a 30 minute pre-ester incubation duration. The difference after a 45-minute incubation was also significant but no more so than at 30 minutes. Therefore a 30 minute pre-ester incubation was selected for later experiments.

4.3.2.3 Varying the time post-quenching before measuring fluorescence

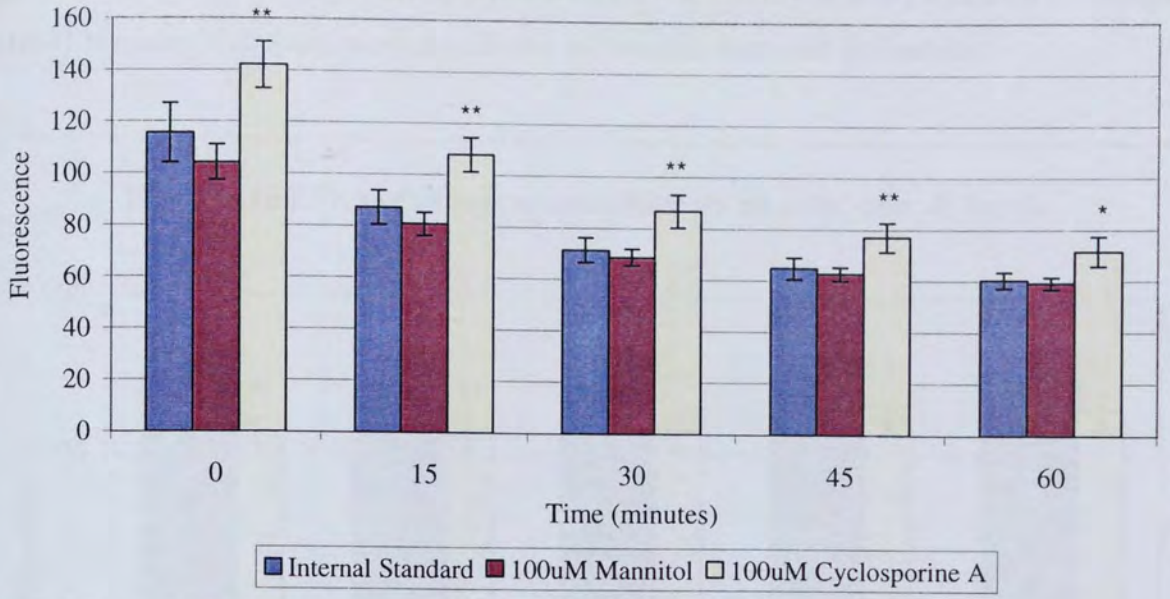
Cobalt has been shown to quench calcein (Figure 4.9 above). However, only the extracellular calcein needs to be quenched not the intracellular. It was hypothesised that cobalt may slowly cross the cell membrane and quench the intracellular calcein. The calculated intracellular calcein would therefore decrease as the time post-quenching before measuring fluorescence increased.

The assay was performed with PBS containing 5 mM glucose (as the internal standard), mannitol (as a negative control), and cyclosporin A (as a positive control). The fluorescence was measured every fifteen minutes post-quenching for one hour, to determine how long after quenching significant results may be achieve.

Figures 4.14, 4.15 and 4.16 below show the effect of time post-quenching on the three different conditions used in the assay. Figure 4.17 will then show the effect of time post-quenching on intracellular calcein relative to the internal standard.

Figure 4.14 shows a clear time-dependent decrease in fluorescence for each of the three sets of data. An ANOVA analysis had a P value of <0.0001 meaning that variation between column means is significantly greater than expected by chance, *i.e.* the results are significantly different from each other. At each time point, there was no significant ($p>0.05$) difference between the fluorescence for the internal standard and for the negative control. However, at every time point the fluorescence for the positive control was significantly greater ($p<0.01$) than the fluorescence for the internal standard.

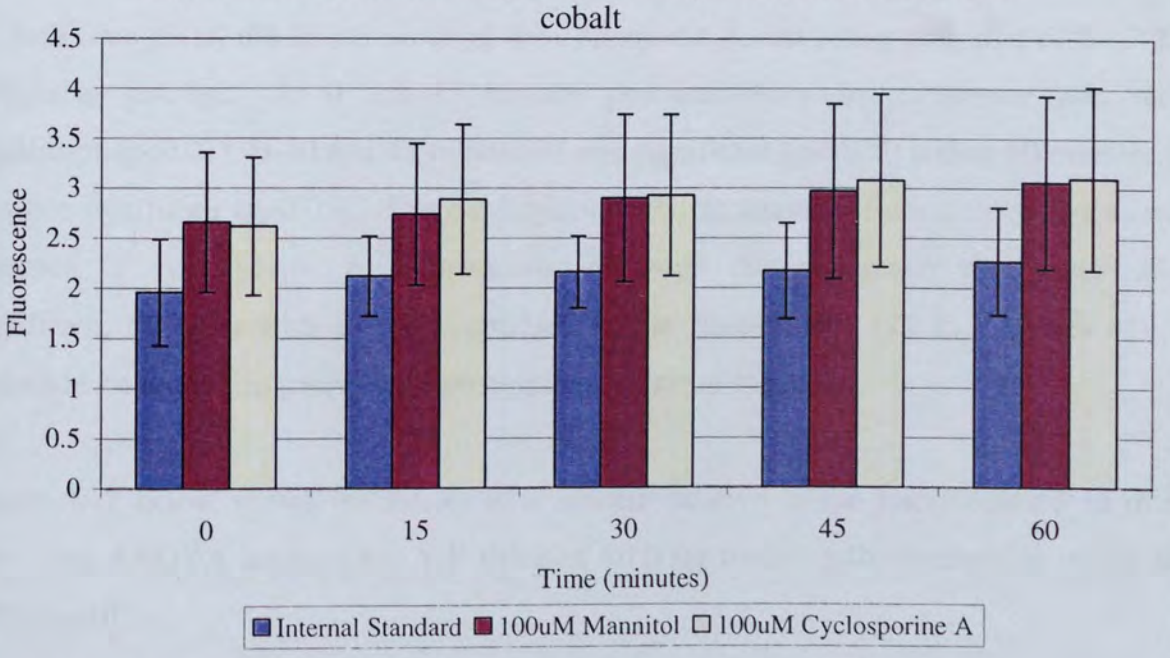
Figure 4.14 Effect of time post quenching on cells, ester & cobalt



Data are mean and standard deviation of the fluorescence four wells.
** Denotes a very significant ($p<0.01$) difference from the internal standard at that time point.

Figure 4.15 below shows the effect of time on the fluorescence of Cells, no ester & no cobalt. This was just a cell suspension so it was not surprising that an ANOVA analysis had a P value of 0.2706 meaning that there was no significant difference between the results. Although the mean fluorescence of the controls was greater than the internal standard at every point, this increase was not significant.

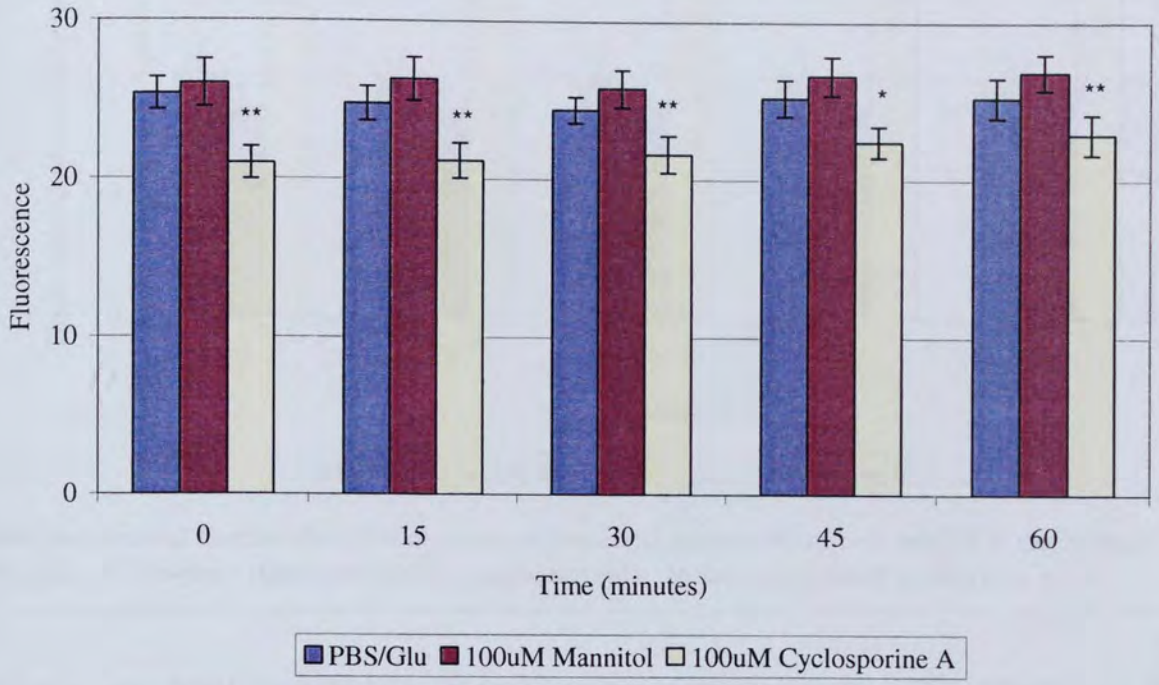
Figure 4.15 Effect of time post quenching on cells, no ester & no cobalt



Data are mean and standard deviation of the fluorescence of four wells.

Figure 4.16 below shows the effect of time on the fluorescence of No cells, ester & cobalt. This was just a suspension of the quenched ester. An ANOVA analysis had a P value of <0.0001 meaning that there were significant differences between the results.

Figure 4.16 Effect of time post quenching on no cells, ester & cobalt

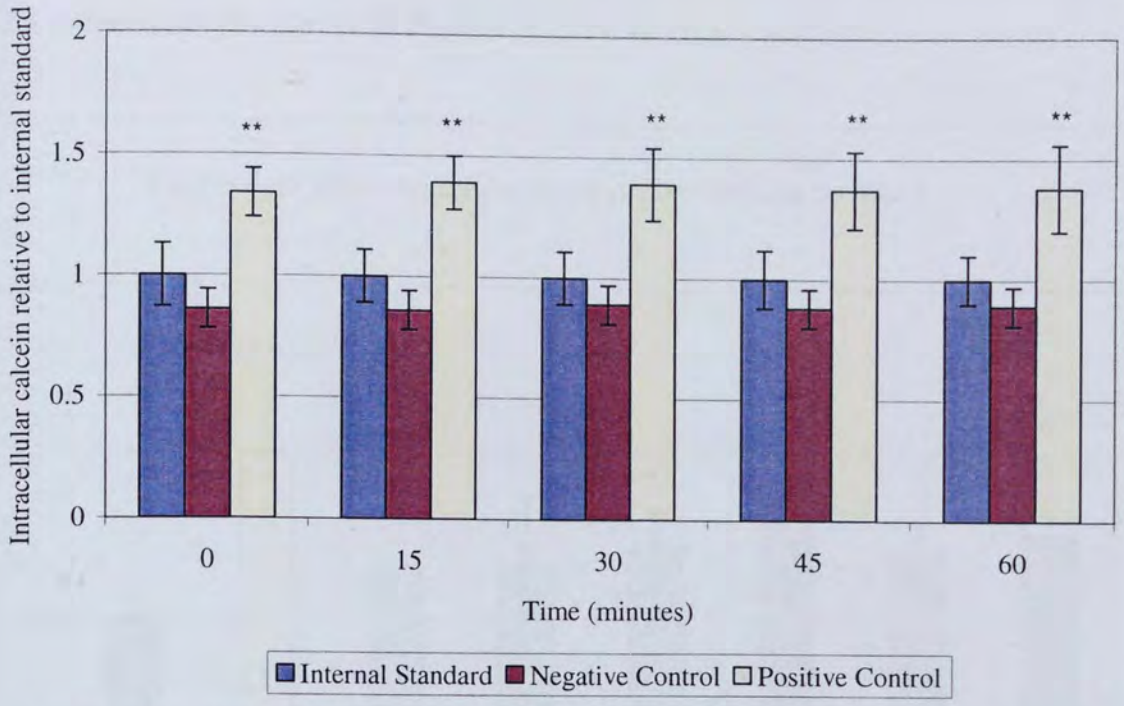


Data are mean and standard deviation of the fluorescence of four wells .
* Denotes a significant ($p<0.05$) difference from the internal standard at each time point.
** Denotes a very significant ($p<0.01$) difference from the internal standard at each time point.

At each time point, the fluorescence of the cyclosporin A was lower than that of the PBS containing glucose. At 0 and 15 minutes post-quenching this difference was very significant ($p<0.01$), at 30 and 45 minutes it was significant ($p<0.05$) and at 60 minutes it was not significant ($p>0.05$). The mechanism of the decrease in fluorescence due to the presence of cyclosporine A is unknown. Although this difference was statistically significant, the difference in the magnitude of the fluorescence (25 to 22) was of no consequence when compared to the changes in the rest of the assay.

Figure 4.17 below shows the intracellular calcein relative to the internal standard over time. An ANOVA analysis had a P value of <0.0001 meaning that variations in results were significant.

Figure 4.17 Effect of time post quenching on intracellular calcein relative to internal standard



Data are mean and standard deviation of results expressed as intracellular calcein relative to the internal standard. ** Denotes a significant ($p<0.01$) difference from the internal standard at each time point.

At every time point the negative control was not significantly ($p>0.05$) different to the internal standard; however, the positive control was significantly ($p<0.01$) greater than the internal standard. While Figure 4.14 (Cells, ester and cobalt) showed fluorescence decreasing over time, Figure 4.14 showed that the differences in fluorescence for each condition stay relatively constant. The intracellular calcein relative to the internal standard calculated after measuring one hour after quenching gave exactly the same results as if it was measured immediately. There was no need to immediately read the quenched fluorescence.

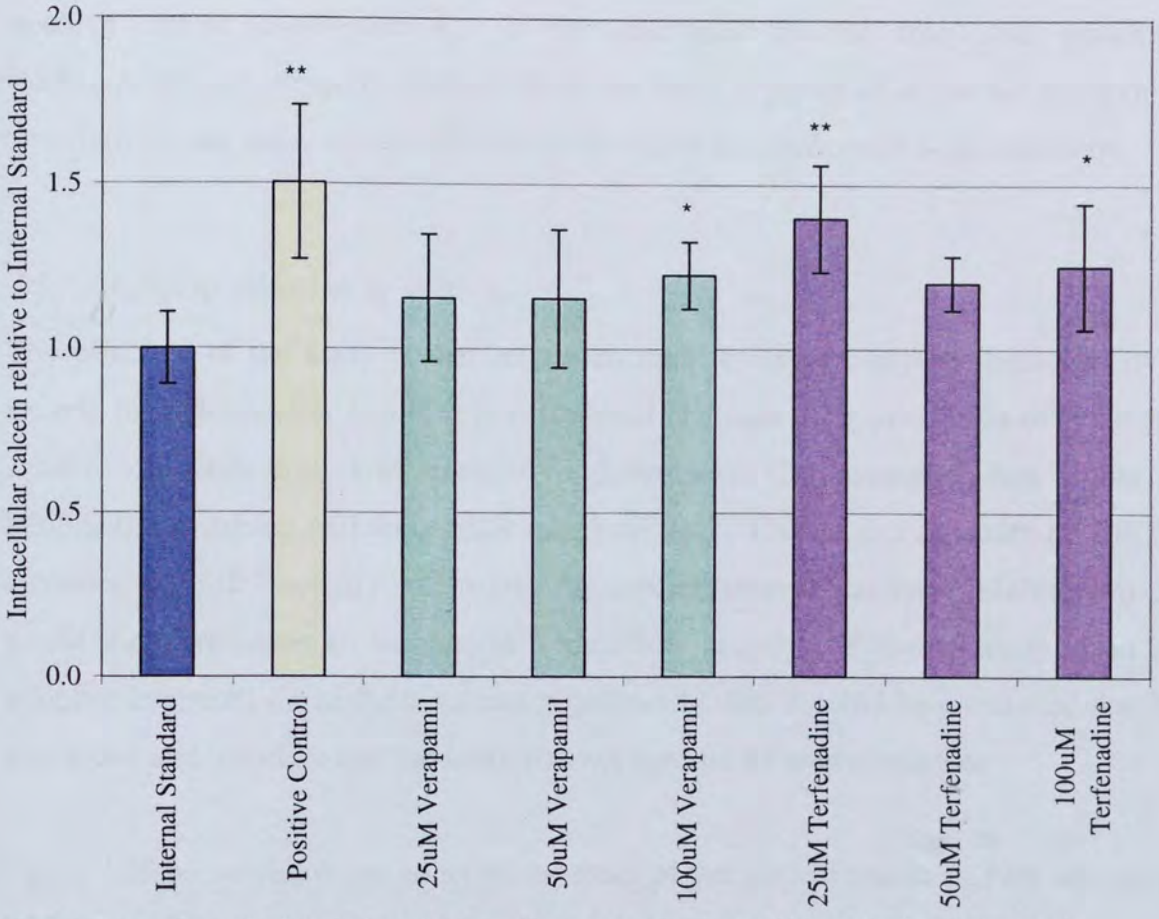
4.3.3 Effect of known inhibitors

4.3.3.1 Effect of inhibitors of p-glycoprotein

Figure 4.18 below shows the effect of the internal standard (PBS containing 5 mM glucose), positive control (cyclosporin A) and various concentrations of two known inhibitors of p-glycoprotein (terfenadine and verapamil) on the assay.

An ANOVA analysis of the data presented in Figure 4.18 had a p value of <0.0001 meaning that the results of the different conditions were significantly different from each other. A Dunnett's post-test was then used to identify any conditions that had significantly different intracellular calcein levels compared to the internal standard.

Figure 4.18 Effect of inhibitors of p-glycoprotein on assay



Data are mean and standard deviation of four wells expressed as intracellular calcein relative to the Internal Standard. * Denotes a significant ($p<0.05$) difference from the internal standard. ** Denotes a very significant ($p<0.01$) difference from the internal standard.

The lowest two concentrations of verapamil (25 μM and 50 μM) were not significantly ($p>0.05$) different from the internal standard, although the higher concentration (100 μM) was significantly ($p<0.05$) greater. These results support the conclusion that verapamil inhibits p-glycoprotein although not as strongly as cyclosporin A does. This agrees with other studies in the literature [Eneroth *et al.*, 2001].

Figure 4.18 above shows that terfenadine significantly increases intracellular calcein at both 25 μM ($p<0.01$) and 100 μM ($p<0.05$) but not at 50 μM ($p>0.05$). There was no

concentration-dependent effect seen, although this may have been hidden by the large standard deviations. It was concluded that terfenadine inhibited p-glycoprotein although not as strongly as cyclosporin A (the positive control) did. This conclusion supported the previously published work [Hait *et al.*, 1993 & Kim *et al.*, 1999] that concluded that terfenadine was an inhibitor of p-glycoprotein.

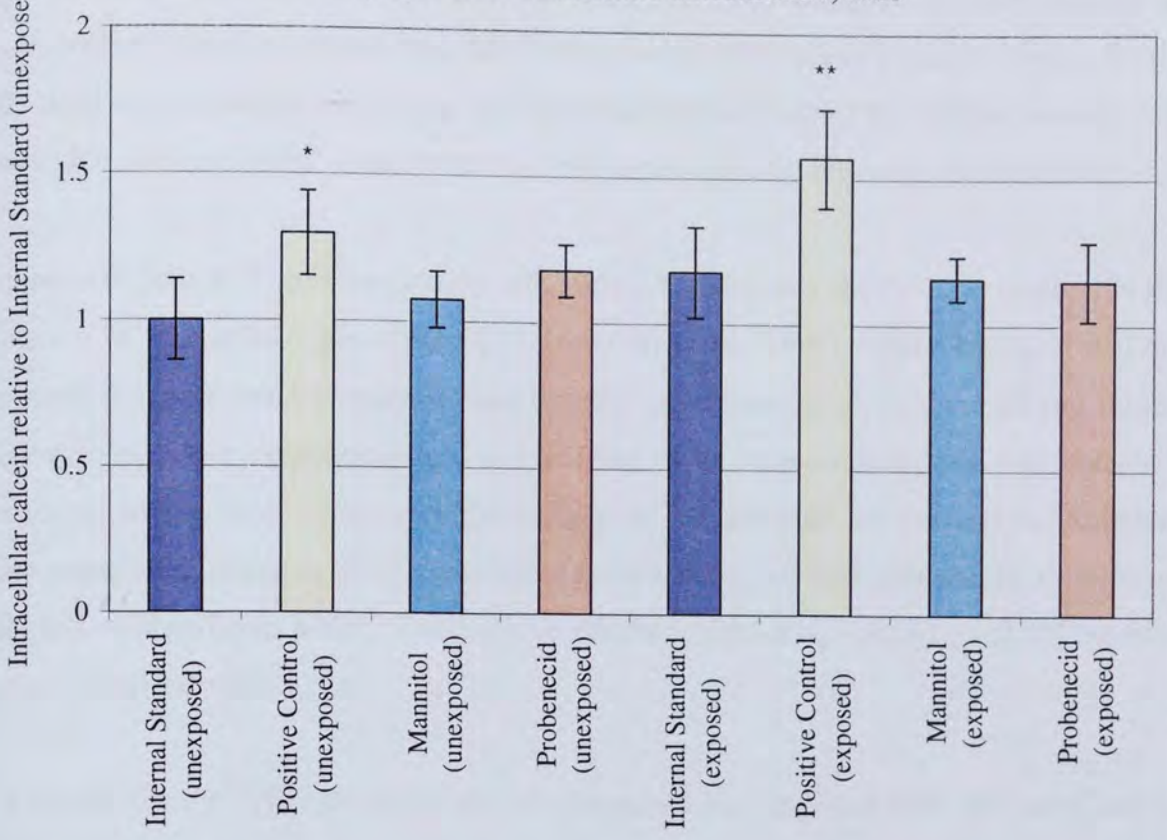
In summary, both known p-glycoprotein inhibitors (verapamil and terfenadine) significantly increased intracellular calcein in the assay, although not as strongly as the positive control (cyclosporin A). It was concluded that the assay does indicate the inhibition of p-glycoprotein activity. However, there is plenty of scope for improving the specificity of the assay *e.g.* the cells could be treated to overexpress p-glycoprotein.

4.3.3.2 Effect of inhibitors of MRP

The principle of the assay is that cells with high levels of p-glycoprotein activity will excrete the calcein ester before it is hydrolysed and thus have low levels of intracellular calcein. In cells with low levels of p-glycoprotein (for example when it has been inhibited), the calcein will accumulate inside the cells. Calcein is a substrate for MRP and therefore any MRP activity will reduce the accumulation of calcein. Inhibitors of MRP would therefore cause an increase in intracellular activity. If the presence of an MRP inhibitor increased intracellular calcein significantly then it could be concluded that MRP was active and therefore that the assay was not specific for p-glycoprotein.

Figure 4.19 below shows the effect on the assay of the internal standard (PBS containing 5 mM glucose), a positive control (cyclosporin A), a negative control (mannitol), and an MRP inhibitor (probenecid) on normal cells and glutathione-depleted cells (which had been exposed to buthionine sulfoximine).

Figure 4.19 Effect on intracellular calcein of cyclosporin A, mannitol and probenecid using normal (unexposed) and glutathione-depleted (exposed to buthionine sulfoximine)cells



Cells were either exposed or not exposed to 75μM buthionine sulfoximine for 24 hours prior to the assay. Data are mean and standard deviation of four wells expressed as intracellular calcein relative to the internal standard (unexposed). * Denotes a significant ($p<0.05$) difference from the internal standard (unexposed). ** Denotes a significant ($p<0.01$) difference from the internal standard (unexposed).

An ANOVA analysis of the data presented in Figure 4.19 above had a p value of 0.0001 meaning that the results of the different conditions were significantly different from each other. A Dunnett's post-test was then used to identify any conditions that had significantly different intracellular calcein levels compared to the internal standard (either unexposed or exposed). In the case of the unexposed cells, the positive control (100 μM cyclosporin A) caused a significant ($p<0.05$) increase in intracellular calcein compared to the internal standard, while mannitol (100 μM) (negative control) had no significant ($p>0.05$) effect. These effects are identical to those shown previously in Figure 4.12.

Probenecid (100 μM) also had no significant ($p>0.05$) effect on intracellular calcein while using unexposed cells, indicating that either there is no MRP activity present or if there is, then it is at such a low level that modulating MRP activity will have no significant effect on intracellular calcein accumulation. The data agrees with a previous study on the effect

of probenecid on the calcein accumulation assay in Caco-2 cells expressing p-glycoprotein [Eneroth *et al.*, 2001]. In that study, probenecid caused an increase in calcein retention at a high concentration (5mM). Although the cells had been designed to only express p-glycoprotein, it was concluded that MRP was present and having a minor effect. As the cells used in the present study have not been selected to overexpress p-glycoprotein, it is reasonable to expect MRP to be present but not necessarily having a significant effect.

Exposure of cells to 75 μM buthionine sulfoximine for 24 hours should have resulted in the depletion of intracellular glutathione [Versantvoort *et al.*, 1995]. MRP transport activity has been shown to need glutathione and therefore depletion of glutathione should inhibit MRP activity. Any inhibition would be identified by an increase in calcein accumulation. Compared to the level of intracellular calcein in the presence of the internal standard (unexposed), the presence of the internal standard, mannitol and probenecid in exposed cells had no significant effect. The positive control caused a significant ($p<0.05$) increase in the unexposed cells.

The results for the different conditions in unexposed and exposed cells are compared in Table 4.4 below. There was no significant difference between the unexposed and exposed cells in the presence of the internal standard, mannitol or probenecid. However exposure to buthionine sulfoximine did cause a significant increase in calcein accumulation in the presence of cyclosporin A.

Table 4.4 Comparison of the significance of the data presented in Figure 4.19 of the effect on intracellular calcein in unexposed and exposed cells in the presence of four different compounds

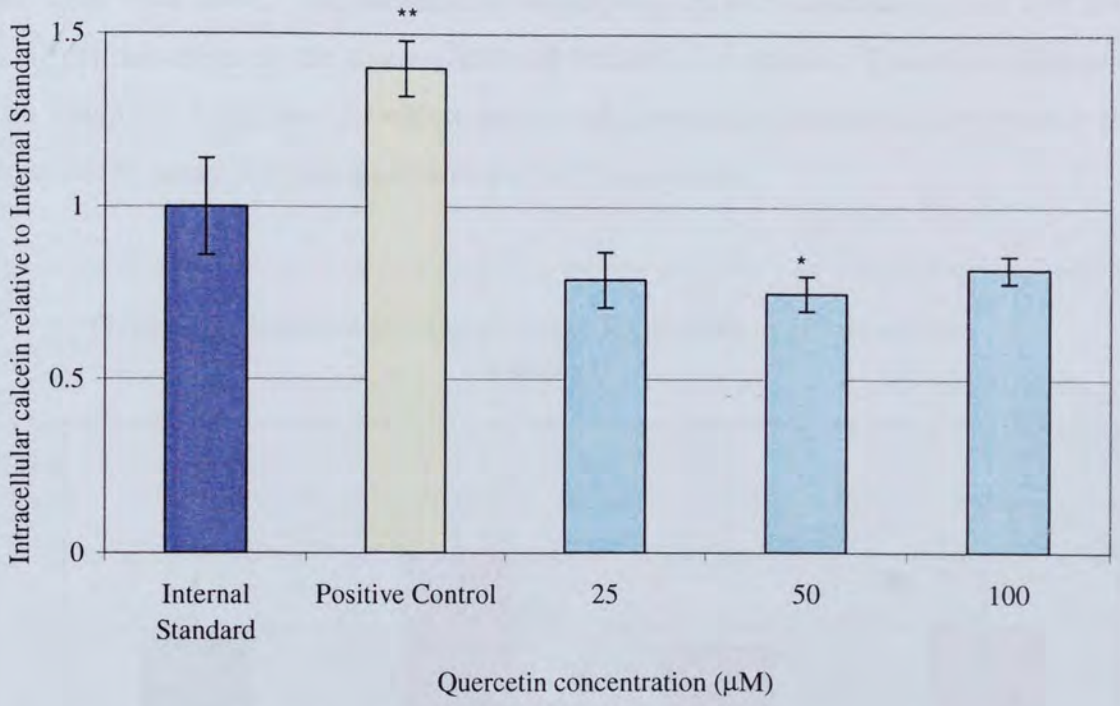
	p value	Significance
Internal Standard (unexposed *vs.* exposed)	>0.05	ns
Cyclosporin A (unexposed *vs.* exposed)	<0.05	*
Mannitol (unexposed *vs.* exposed)	>0.05	ns
Probenecid (unexposed *vs.* exposed)	>0.05	ns

Significance calculated using a Bonferroni Multiple Comparisons Test of the data. Significant differences are highlighted in blue.

Exposure of normal cells to buthionine sulfoximine should inhibit a constant amount of MRP activity and, therefore, should increase calcein accumulation by a constant amount. Exposure had no significant effect in the presence of the internal standard, negative control (mannitol) or a known MRP inhibitor (probenecid) but there was a significant increase in the presence of cyclosporin A.

Figure 4.20 below shows the effect of an MRP inhibitor (quercetin) compared to the internal standard (PBS containing 5mM glucose) and the positive control (cyclosporin A) on the assay.

Figure 4.20 The effect of an MRP inhibitor (Quercetin) on the assay



Data are mean and standard deviation of four wells expressed as intracellular calcein relative to the internal standard. * Denotes a significant ($p<0.05$) difference from the internal standard. ** Denotes a very significant ($p<0.01$) difference from the internal standard.

Quercetin only significantly altered the intracellular calcein level at a concentration of 50 μM and that was a reduction not an increase. As noted above, a previous study found that 200 μM quercetin (and other flavonoids) significantly reduced Rhodamine-123 accumulation in large-cell lung MRP-expressing cells unlike other modulators of MRP activity (*e.g.* buthionine sulphoximine) [Versantvoort *et al.*, 1996]. It was proposed that the flavonoids depolarise the membrane potential and accelerate the efflux of the dye from the cells. If the quercetin was accelerating the efflux of the calcein then there would be a

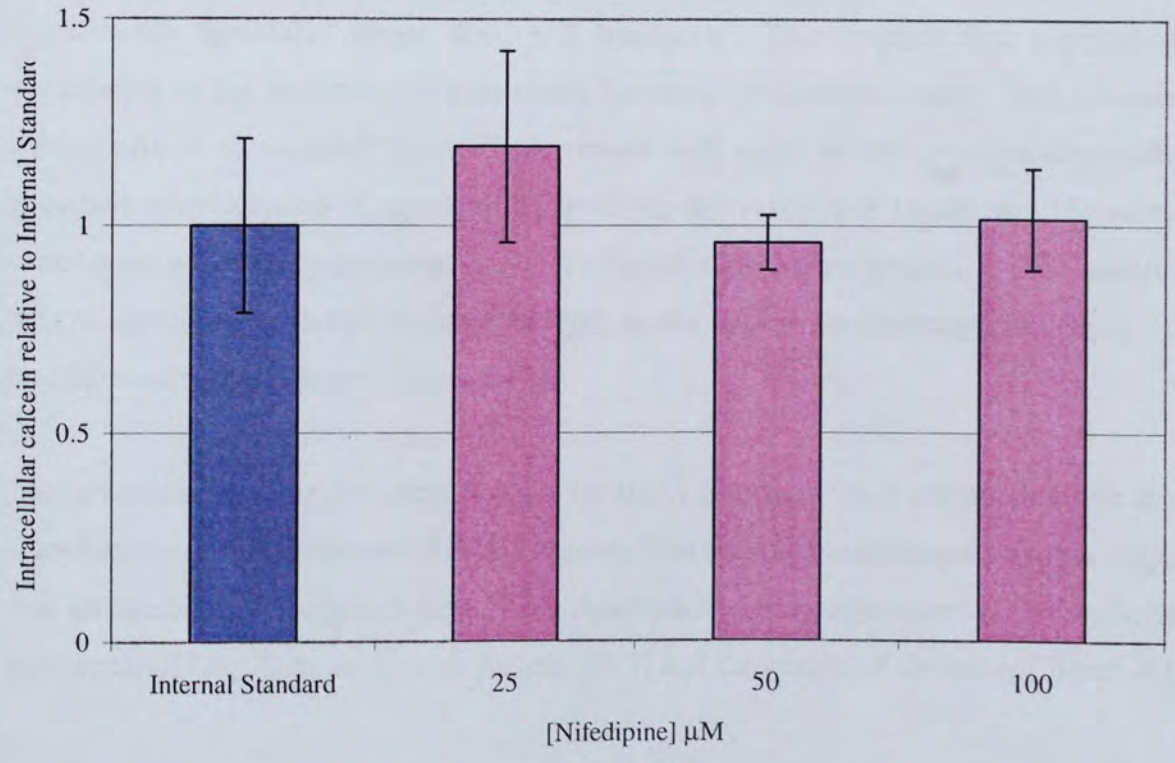
concentration-dependent reduction in intracellular calcein, which is not seen in Figure 4.20 above.

In summary, inhibition of MRP by probenecid, buthionine sulfoximine or quercetin did not cause a significant effect on intracellular calcein accumulation. It was concluded that the assay did not detect changes in MRP activity.

4.4.3.3 Effect of an inhibitor of neither p-glycoprotein or MRP

Figure 4.21 below shows the effect on the assay of nifedipine, a substrate for CYP3A4 but not p-glycoprotein. A one-way ANOVA statistical test on the data had a p value of 0.29, considered not significant, *i.e.* the results of the different conditions are not significantly different from each other. The presence of nifedipine, up to a concentration of 100 μM, has no significant effect on the accumulation of intracellular calcein. Therefore nifedipine is not an inhibitor of calcein-AM efflux and as the previous experiments have shown the specificity of the assay, it is not an inhibitor of p-glycoprotein.

Figure 4.21 Effect of nifedipine on the accumulation of intracellular calcein



Data are mean and standard deviation of four wells expressed as intracellular calcein relative to the Internal Standard. There was no significant difference between the four conditions.

The assay results support the conclusions of the studies that found that nifedipine could not restore doxorubicin sensitivity to resistant mouse leukaemia cells [Ramu *et al.*, 1984] or affect the transport of daunorubicin or digoxin in porcine kidney cells [Katoh *et al.*, 2001].

In summary, a majority of previous studies have found that nifedipine is not an inhibitor of p-glycoprotein and this study found that nifedipine had no effect on the assay.

4.3.4 Comparing the assay to transepithelial transport

The activity of p-glycoprotein is to pump vincristine across the apical surface and out of the cells. This activity hinders apical-basolateral (A-B) transport and promotes basolateral-apical (B-A) transport. Inhibitors of p-glycoprotein should therefore cause an increase in A-B transport and a reduction in B-A transport.

The transepithelial transport of vincristine sulphate was studied in Caco-2 cells. The apparent permeability (Papp) was determined in both directions (A-B and B-A) in the presence and absence of cyclosporin A, verapamil, buthionine sulfoximine, quercetin or nifedipine. The results are shown in Table 4.5 below.

It can be seen that in the absence of any inhibitor, B-A transport of vincristine was significantly ($p=0.001$) larger than A-B transport. This implies that p-glycoprotein contributes in the formation of a transport barrier to vincristine influx. The presence of cyclosporin A significantly ($p<0.05$) increased A-B while in the opposite direction, the presence of cyclosporin A significantly ($p<0.01$) decreased B-A transport. The resulting conclusion was that cyclosporin A was an inhibitor of p-glycoprotein . This conclusion was in agreement with the literature on cyclosporin A [*e.g.* Spatzenegger & Jaeger, 1995] and the results of the assay (Figure 4.12).

The presence of verapamil significantly ($p<0.01$) increased A-B transport while it also significantly ($p<0.01$) decreased B-A transport. The resulting conclusion was that verpamil was an inhibitor of p-glycoprotein. This conclusion was in agreement with the literature on verapamil [*e.g.* Spatzenegger & Jaeger, 1995] and the results of the assay (Figure 4.18).

Table 4.5 Apparent permeability coefficients of vincristine in presence and absence of Cyclosporin A, Verapamil, Buthionine Sulfoximine, Quercetin or Nifedipine

Direction	Inhibitor	Papp x10^{6} [a] (cm/sec/mg protein)	Significance[b] p
Apical-Basolateral	None	2.10±0.49	
	100 μM cyclosporin A	6.31±2.19	<0.05
	100 μM verapamil	5.76±0.69	<0.01
	100 μM buthionine sulfoximine	1.19±1.11	>0.05
	100 μM quercetin	2.25±1.54	>0.05
	100 μM nifedipine	4.99±1.05	<0.05
Basolateral-Apical	None	11.7±1.93	
	100 μM cyclosporin A	4.99±1.02	<0.01
	100 μM verapamil	4.58±1.82	<0.01
	100 μM buthionine sulfoximine	10.3±3.75	>0.05
	100 μM quercetin	9.38±0.38	>0.05
	100 μM nifedipine	12.7±3.25	>0.05

[a] Calculated Papp values are expressed as mean ± standard deviation, n=3

b An two-tailed t-test was performed on the results for each direction. . Statistically significant results are highlighted in blue.

The presence of buthionine sulfoximine had no significant ($p>0.05$) effect on the transport of vincristine in either direction. The resulting conclusion was that buthionine sulfoximine was not an inhibitor of p-glycoprotein. This conclusion was in agreement with the literature on verapamil [*e.g.* Versantvoort *et al.*, 1995] and the results of the assay (Figure 4.19).

The presence of quercetin had no significant ($p>0.05$) effect on the transport of vincristine in either direction. The resulting conclusion was that buthionine sulfoximine was not an inhibitor of p-glycoprotein. This conclusion was in agreement with the results of the assay (Figure 4.20).

The presence of nifedipine had a significant ($p<0.05$) effect on the transport of vincristine in one direction but not the other. The resulting conclusion was that nifedipine may or may not be an inhibitor of p-glycoprotein. The results do, however, contradict the conclusion of

the study that found that nifedipine inhibited the basolateral-to-apical transport of digoxin in Caco-2 cells and therefore inhibited p-glycoprotein [Cavet *et al.*, 1996]. A majority of previous studies have found that nifedipine was not an inhibitor of p-glycoprotein, this study found that nifedipine had no effect on the assay but did on the transport if vincristine. It was concluded that nifedipine is not an inhibitor of p-glycoprotein yet some uncertainties remain.

4.4 Conclusion

The optimum conditions for the calcein-AM assay were identified. The optimum pH was found to be pH 7. There was a clear dose-dependent reduction in calcein fluorescence in the presence of an increasing cobalt chloride concentration, therefore it was concluded to add 20 μl of 2 mM cobalt chloride to quench the extracellular calcein in the assay. There was a linear relationship between the concentration of calcein and the fluorescence at the measured wavelength over a range of concentrations (0-0.1 μM). It was also concluded that DMSO and ethanol could be used as co-solvents up to a concentration of 5%.

Two modifications to the calcein accumulation assay were proposed: quenching with cobalt chloride and preincubating the test solutions with the cells. Cobalt chloride has clearly been shown to quench the fluorescence of calcein. It can be used to quench extracellular calcein to allow the measurement of intracellular calcein, removing the need for a plate centrifuge. The trade off for removing the washing stages is the requirement to measure intracellular calcein in the three different conditions (cells, ester and cobalt, no cells, no ester and no cobalt, and no cells, ester and cobalt). The problem is the fluorescence of the no cells, ester and cobalt relative to the fluorescence of cells, ester and cobalt (approximately 20%). This is a significant proportion although the consistency of this value means it is subtracted form itself when calculating intracellular calcein. The assay needs to be refined to reduce the fluorescence of this control (for example using a different concentration of ester or different post-ester incubation duration).

The second modification was a preincubation stage, which increased the significance of the difference between the internal standard and positive control. The two modifications to the protocol were beneficial due to an increase in efficiency and a reduced need for special equipment.

The effect on intracellular calcein was identical for the internal standard and negative control (mannitol) however the positive control (cyclosporin A) significantly increased intracellular calcein. Two other known inhibitors of p-glycoprotein (verapamil and terfenadine) were tested and their potencies compared to cyclosporin A, used as a positive control. They all significantly increased intracellular calcein. The order of potencies were cyclosporin A > terfenadine > verapamil. This order correlates well with previously published studies. The assay detected inhibitors of p-glycoprotein and allowed their potencies to be ranked. However, the assay was dependent on the concentration of the inhibitors.

Probenecid, a known inhibitor of MRP but not p-glycoprotein, did not cause a significant effect on intracellular calcein. Exposure to buthionine sulfoximine also inhibited MRP through glutathione-depletion. This also had no significant effect. However, the combined effects of exposure to buthionine sulfoximine and the presence of cyclosporin A caused a more significant increase in calcein accumulation than cyclosporin A alone. The presence of quercetin caused a significant decrease in intracellular calcein at one concentration. The lack of increase indicated the confusing results previously seen with this and related compounds. It was concluded that MRP plays a minor non-significant role in the assay.

Nifedipine is an inhibitor of CYP3A4 but not p-glycoprotein or MRP. It had no significant effect on calcein accumulation up to a concentration of 100 μM. Nifedipine acts as a negative control; no effect was expected or identified.

The modified assay was found to be specific for p-glycoprotein rather than MRP. The final protocol for the assay included the two modifications previously mentioned and included an internal standard (PBS containing 5 mM glucose) and a positive control (100 μM cyclosporin A) on each 96-well plate.

The transport of vincristine sulphate in the presence and absence of a range of compounds was studied. The apparent permeability (Papp) values produced were analysed and the inhibitory effect of cyclosporin A, verapamil and nifedipine (in one direction only) but not buthionine sulfoximine or quercetin were identified. The assay produced the same identification as the transport experiments with the exception of nifedipine.

The modified protocol now allows the identification of the effect of compounds (in solution) on the activity of p-glycoprotein using a simple cell suspension instead of growing cell monolayers. The experiments in this chapter proved that the modified calcein accumulation assay specifically identifies inhibitors of p-glycoprotein as MRP activity is present but not significant. The assay can be used as a screen for inhibitory activity with other more time-consuming techniques then used to quantify the relative size of the effect. There are a number of potential future modifications to improve the efficiency and usefulness of the assay. These include using cells that have been modified to express high levels of p-glycoprotein to increase the difference between the positive control and the internal standard and automating the system to reduce variations due to human error. Studies could also be performed to monitor the expression of p-glycoprotein and MRP on different days to identify the optimum day to perform the assay. If the protocol is altered in the future then the panel of experiments performed here can be repeated to confirm the specificity of the assay.

5. INVESTIGATING P-GLYCOPROTEIN: EFFECT OF FRUIT JUICES, THEIR COMPONENTS AND OTHER NATURAL COMPOUNDS

ABSTRACT:

Previously published studies have found that a range of natural products and compounds affect the absorption of many drugs. The effect on the modified calcein assay of grapefruit juice and other fruit juices and oils was investigated to identify any inhibition of the activity of p-glycoprotein. The clinically relevant buffering of grapefruit juice to pH 7 led to a four-fold increase in intracellular calcein and hence significant inhibition of p-glycoprotein. Buffered orange and lemon juices had no effect on the assay. Emulsions (1%) of grapefruit and bergamot oils significantly increased the level of intracellular calcein, indicating the presence of p-glycoprotein inhibitors among their constituents. Flavone derivatives had previously been found to be inhibitors of CYP3A4 yet neither naringin nor naringenin had any significant effect at concentrations found in grapefruit juice. Of the other (non-grapefruit) flavone derivatives tested, hesperidin, found in orange juice, had no significant effect; kaempferol and rutin also had no effect while genistein significantly inhibited p-glycoprotein (results that support previous studies). Hydroxycinnamic acids had no effect on p-glycoprotein. Studies on other compounds found that the balance between inhibiting p-glycoprotein and disrupting cell membranes depends on the compound containing an oxygen atom and the size of the negative charge on it, as well as three-dimensional arrangement of the atoms.

5.1 Introduction

A range of natural products and compounds have been found to affect the absorption of drugs (see Section 1.9). The constituents of fruit juices and oils, their activity and the activity of related compounds are examined below.

5.1.1 Grapefruit juice

Grapefruit juice is composed of many compounds, the most abundant being in aqueous solution. The chemical characteristics of the volatile components of fruit juices have been used to compare hybrid fruit with their parent fruits [Shaw *et al.*, 2001]. Table 5.1 below lists compounds found in a Pearl tangelo grapefruit (*Citrus paradisi* Macfadyen).

Table 5.1 Concentration of volatile components in juice of Pearl tangelo grapefruit (*Citrus paradisi* Macfadyen) [adapted from Shaw *et al.*, 2001]

Compound	ppm
Ethanol	75.6
Limonene	20.8
Methanol	12.1
(E)-Ocimene	11.4
Myrcene	1.06
α-Pinene	0.793
Octanol	0.47
Valencene	0.35
(Z)-3-Hexenol	0.2
Caryophyllene	0.16
Linalool	0.13
Ethyl acetate	0.071
(E)-Linalool oxide	0.052
Propanol	0.043
α-Phellandrene	0.033
1-Penten-3-ol	0.032
Sabinene	0.029
Hexanol	0.024
Hexanal	0.018
Ethyl propionate	0.015

Compound	ppm
1-Penten-3-one	0.013
2-Pentanol	0.012
3-Methylbutanol	0.011
Heptanal	0.007
Decanal	0.006
Nonanal	0.005
Perillaldehyde	0.004
Carvone	0.003
Pentanol	0.002
2-Methylpropanol	0.0001
Butanol	0.0001
Methyl butanoate	0.0001
3-Methyl-2-butenol	0.0001
(E)-2-hexenal	0.0001
Ethyl hexanoate	0.0001
Octanal	0.0001
γ-Terpinene	0.0001
Ethyl octanoate	0.0001
4-Terpineol	0.0001

Of the compounds listed, ethanol has previously been studied (Figure 5.7) and limonene, myrcene and α-pinene are examined amongst others below.

5.1.2 Other juices and oils

5.1.2.1 Orange juice

The effect of orange juice on the formation of 6β-hydroxytestosterone was studied in a human lymphoblastoid cell line (h3A4/OR) [Baltes *et al.*, 2001]. CYP3A4 activity was 99% (± 2%) control activity in the presence of orange juice. This compares to 18% (± 4%) in the presence of grapefruit juice. It was concluded that orange juice is not an inhibitor of CYP3A4.

Human myelogenous leukaemia K562 cells and their adriamycin-resistant variant K562/ADM were used to study the effect of ethyl acetate extracts of orange and grapefruit juices on vincristine uptake [Ikegawa *et al.*, 2000]. The resistant cells accumulated a significantly lower (4.2 *vs.* 92.4 μl/mg protein) amount of vincristine. Both extracts significantly increased the uptake (by inhibiting efflux) in to the cells (to 3 and 7 fold for grapefruit and orange juice respectively). The extracts consist of a number of components including the methoxyflavones tangeretin, nobiletin and heptamethoxyflavone. A previous study of the cold pressed peel oil of oranges using gas chromatography and mass spectrometer methods [Stremple, 1998] discovered six methoxyflavones, hexamethoxyflavone, nobiletin, tetra-O-methylscutellarein, tangeretin, sinensetin and heptamethoxyflavone.

The three compounds in the ethyl acetate extract (tangeretin, nobiletin and heptamethoxyflavone) significantly increased vincristine uptake in a concentration-dependent manner. Compared to verapamil, their relative potencies were 0.47 for tangeretin, 1.02 for heptamethoxyflavone and 1.17 for nobiletin. Cyclosporin A had a relative potency of 1.02. These methoxyflavones, components of orange juice, strongly inhibit p-glycoprotein although the results may not be clinically relevant.

5.1.2.2 Lemon juice

Chiral capillary electrophoresis has been used to identify characteristic flavone glycosides found in lemon juice [Gel-Moreto *et al.*, 2001]. Both diastereomers of hesperidin and eriocitrin were identified in the juice.

The oxidative stress of active oxygen and free radicals attacking fatty acids in cell membranes have been associated with a number of diseases. Diabetes mellitus is associated with increased lipid peroxidation and increased oxidative stress. Oxidative stress was induced in rats using streptozocin [Miyake *et al.*, 1998], which is toxic to β-cells and induces diabetes mellitus. The protective effect of a crude mixture of flavonoids from lemon juice was then determined. The mixture reduced lipid oxidation in the liver, kidney, and serum by scavenging free radicals. It was concluded that dietary flavonoids might play a role in preventing the development of diabetes and other diseases caused by oxidative stress.

The effect of lemon juice on the formation of 6β-hydroxytestosterone was studied in a human lymphoblastoid cell line (h3A4/OR) [Baltes *et al.*, 2001]. Lemon juice reduced CYP3A4 activity to 40% (± 8%) control, which was as potent as 24 μM quercetin. This compares to 18% (± 4%) in the presence of grapefruit juice. Further studies showed that grapefruit juice inhibition is a combination of competitive and non-competitive inhibition, while lemon juice inhibition is purely competitive. It was concluded that the juice components responsible for inhibiting CYP3A4 are different in lemon and grapefruit juices.

5.1.2.3 Grapefruit oil

The major constituent of grapefruit oil is limonene (64-85%) [Pino & Sanchez, 2000]. Other constituents found in grapefruit oil included geraniol, citral, citronellal and neral. The cold pressed peel oil of grapefruits has been studied using gas chromatography and mass spectrometer methods [Stremple, 1998]. Three methoxyflavones were discovered, hexamethoxyflavone, nobiletin, and tangeretin, at much lower concentrations than they are found in orange oil.

Grapefruit oil is extracted from grapefruit peel and contains some of the constituents of grapefruit juice concentrated up to three orders of magnitude. Human MCF-7 cells

expressing p-glycoprotein treated with grapefruit oil had an increased accumulation of intracellular doxorubicin compared to untreated cells [Abedel-nasser *et al.*, 2000].

5.1.2.4 Bergamot oil

Bergamot oil is extracted from bergamot fruit (*Citrus bergamia*) and is an important constituent of many fragrance materials. A number of its components have been isolated include bergapten (5-methoxypsoralen) (0.37% in oil) and its breakdown product limettin (5,7-dimethoxypsoralen) [Croud *et al.*, 1983]. Another study found forty-seven components including limonene (36.2%), linalyl acetate (36.3%), linalool (6.9%), γ-terpinene (5.9%), β-pinene (3.9%), and myrcene (1.3%) [Kirbaslar *et al.*, 2001].

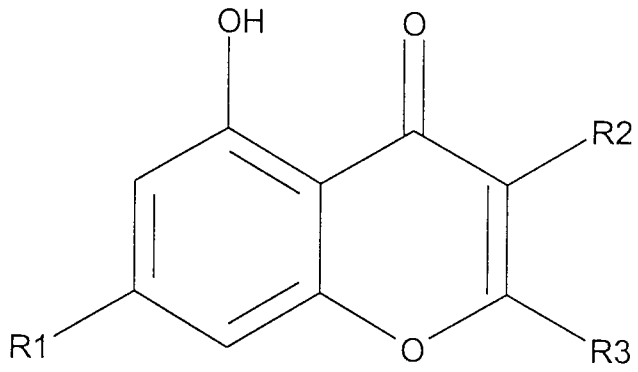
5.1.3 Flavone derivatives

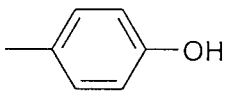
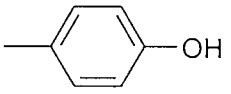
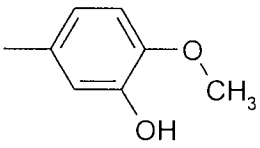
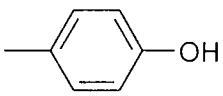
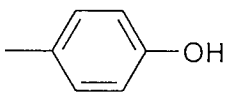
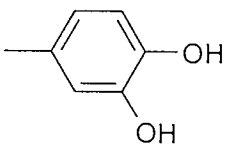
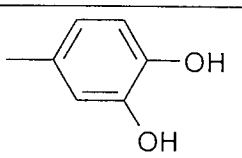
Flavonoids are a large group of naturally occurring, low molecular weight, polyphenolic compounds widely distributed in the plant kingdom as secondary metabolites. About 500 varieties of flavonoids are known. They are based on the parent compound flavone (2-phenyl benzopyrone). Flavonoids occur in practically all parts of plants including fruit, vegetables, nuts, seeds, leaves, flowers and bark. The beneficial effects of preparations that contain flavonoids have been known for centuries. Some symptoms originally thought to be due to vitamin C deficiency such as bruising due to capillary fragility were found in early studies to be relived by crude vitamin C extract but not by purified vitamin C. The bioflavonoids, formally called 'vitamin P', were found to be the essential components in correcting this bruising tendency and improving the permeability and integrity of the capillary lining. These bioflavonoids included hesperidin, citrin, rutin, flavones, flavonols, catechin and quercetin [Garg *et al.*, 2001].

Phenolic compounds can act as free radical scavengers by virtue of their hydrogen-donating ability, forming aryloxyl radicals. Other functional groups in the structure can enhance the antioxidant activity by stabilising the aryloxyl radicals. Therefore the flavone derivatives have various levels of antioxidant activity.

The structures of some flavone derivatives are shown in Figure 5.1 below.

Figure 5.1 The structure of the flavone skeleton and some flavone derivatives



Name	R1	R2	R3
Naringenin	—OH	—H	
Naringin	Rhamnosido-glucoside sugar residue	—H	
Hesperidin	Rhamnosido-glucoside sugar residue	—H	
Genistein	—OH		—H
Kaempferol	—OH	—OH	
Quercetin	—OH	—OH	
Rutin	—OH	Rutinoside sugar residue	

5.1.3.1 Naringenin

The structure of naringenin is shown in Figure 5.1 above. Naringenin is found in grapefruit juice at various levels: 10-20 mg/L (~55 μM) in one study [Ho *et al.*, 2000], ~241 mg/L (~885 μM) in another [Ameer *et al.*, 1996]. It is also found in orange juice (~18 mg/L, ~66 μM) [Ameer *et al.*, 1996]. It is thought to be formed from naringin in the intestine after oral administration [Ameer *et al.*, 1996] where intestinal enzymes cleave the rhamnosido-glucoside sugar residue from naringin to leave the aglycone naringenin.

In vitro studies showed that naringin was a less potent inhibitor of CYP3A4 activity than naringenin [Guengerich & Kim, 1990]. A series of experiments examined if naringin and naringenin were the primary CYP3A4 inhibitors in grapefruit juice [Edwards & Bernier, 1996]. Naringenin did not form during the experiment so did not contribute to the *in vitro* inhibitory effect. It was concluded that naringenin was not primarily responsible for the *in vitro* inhibition of CYP3A4 by grapefruit juice [Edwards & Bernier, 1996].

There have been a few studies examining the effect of naringenin on p-glycoprotein. Naringenin reduced the accumulation of adriamycin (to 77% control) in HCT-15 colon cells [Critchfield *et al.*, 1994]. It was concluded that naringenin upregulates the activity of p-glycoprotein. A hexahistidine-tagged C-terminal nucleotide-binding domain (H_6-NBD2) from mouse p-glycoprotein was overexpressed and purified [Conseil *et al.*, 1998]. The protein had intrinsic fluorescence due to the presence of a tryptophan residue but the binding of any compounds quenches the fluorescence allowing an IC_{50} to be calculated. Naringenin had an IC_{50} of 28.5 μM which meant that it bound but weakly compared to other compounds [Conseil *et al.*, 1998]. Naringenin weakly binds to p-glycoprotein and possible has an activating effect.

In summary, naringenin is possibly the compound that has the major effect on CYP3A4 *in vivo* when grapefruit juice is consumed, however, it has not been shown to have any major inhibiting effect on p-glycoprotein

5.1.3.2 Naringin

The structure of naringin is shown in Figure 5.1 above. Naringin is the main bitter component in grapefruit juice. Its concentration in juice varies between 115 to 384 mg/L (~200-660 μM) after hand-squeezing fresh grapefruit and up to 703 mg/L (~1205 μM) in

commercially sold juices [Ho *et al.*, 2000]. Another study [Ameer *et al.*, 1996] found naringin in grapefruit juice (~373 mg/L) but not in orange juice.

The effect of naringin, rutin and catechin on xanthine oxidase activity, lipid peroxidation, DNA cleavage and their free radical scavenging capacity was investigated *in vitro* and using rat liver microsomes where necessary [Russo *et al.*, 2000]. Rutin and catechin were more potent than naringin at inhibiting superoxide anion formation, quenching DPPH and inhibiting lipid peroxidation. All three flavonoids inhibited the activity of xanthine oxidase, a source of superoxide anions, and reduced DNA damage caused by exposure to UV light. It was concluded that naringin, rutin and cetechin all have antioxidant properties.

In vitro studies showed that naringin was a less potent inhibitor of CYP3A4 activity than its aglycone naringenin [Guengerich & Kim, 1990]. A series of experiments examined if naringin was the primary CYP3A4 inhibitors in grapefruit juice [Edwards & Bernier, 1996]. An aqueous solution of naringin at the same concentration as that found in grapefruit juice produced a small decrease in CYP3A4 activity. Dilution of juice with naringin solution reduced the inhibitory effect of the juice although the naringin concentration remained constant. An organic extract of juice had considerable inhibitory effect although it did not contain naringin. It was concluded that naringin was primarily responsible for the *in vitro* inhibition of CYP3A4 by grapefruit juice.

As naringin exists as two optical isomers, one explanation for the lack of effect of naringin was that the active isomer was at a much lower concentration compared to the inactive isomer. However, a study of (+)-naringenin and (-)-naringenin showed only a slight difference in their potencies [Bailey *et al.*, 2000]. Therefore naringin, at concentrations found in grapefruit juice, does not produce the clinical effect like that of grapefruit juice. Naringin reduced the accumulation of adriamycin (to 88% control) in HCT-15 colon cells [Critchfield *et al.*, 1994]. It was concluded that naringin upregulates the activity of p-glycoprotein.

In summary, naringin is the main bitter component in grapefruit juice. It has been shown to have antioxidant properties but has no effect of CYP3A4 activity and only a minor activating effect on p-glycoprotein. It is not responsible for the effect of grapefruit juice.

5.1.3.3 Hesperidin

The structure of hesperidin is shown in Figure 5.1 above. It is a flavone glycoside comprising of an aglycone, hesperitin, and an attached disaccharide, rutinose (rhamnosido-glucoside). It is the primary flavonoid found in orange juice (~65 mg/L) but is also found in grapefruit juice (~6.5 mg/L) [Ameer *et al.*, 1996].

Hesperidin was first discovered in 1827, by Lebreton, but not in a pure state. It is an abundant and inexpensive by-product of citrus cultivation and is the major flavonoid in sweet orange and lemon. A mixture of hesperidin and eriodictyol, called 'citrin' or 'vitamin P', was considered to possess a vitamin-like activity, decrease capillary permeability and fragility, reduce the signs of hypovitaminosis C, and have antioxidant-dependent vitamin C sparing activity. Hesperidin deficiency has been linked with abnormal capillary leakiness and pain in the extremities causing aches, weakness and night leg cramps. Supplemental hesperidin has also been found to reduce oedema, reduce hypotension, reduce plasma cholesterol, block calcium channels, inhibit platelet and cell aggregation, regulate oestrogen levels, and have anti-inflammatory, antimicrobial, antifertility, anticarcinogenic, free radical scavenging, antioxidant, analgesic and antiallergic abilities [Garg *et al.*, 2001].

The metabolic fate of hesperidin has been studied in rats and humans. The major urinary products formed are different in both species but are formed from the splitting of the aglycone [Garg *et al.*, 2001]. Hesperidin was found to be transformed to its aglycone, hesperitin, in the intestine by bacteria producing α-rhamnosidase, β-glucosidase or endo-β-glucosidase. Hesperitin can then be absorbed as a free compound or a glucuronate-conjugate.

The oral absorption of hesperidin from citrus products has been examined [Ameer *et al.*, 1996]. It was absorbed but had a low bioavailability (24%). The aglycone hesperitin was detected in both urine and plasma. Citrus flavonoids were thought to undergo glucuronidation before urinary excretion. Intestinal permeability to hesperidin glycosides was investigated using Caco-2 cells [Kim *et al.*, 1999]. Hesperidin did not permeate, probably due to its low solubility. The glycosides did permeate in a time- and dose-dependent manner via a paracellular pathway.

Hesperidin reduced the accumulation of adriamycin (to 88% control) in HCT-15 colon cells [Critchfield *et al.*, 1994]. It was concluded that hesperidin upregulates the activity of p-glycoprotein. In another study, the uptake of [^{3}H]-vincristine across the blood-brain barrier was increased by the aglycone hesperitin but not hesperidin [Mitsunaga *et al.*, 2000]. The effect was thought to be due to the stimulation of p-glycoprotein. Hesperidin has also been found to interact with daunomycin [Melzig *et al.*, 1997].

Hesperidin needs to be metabolised (to hesperitin) before it can cross a Caco-2 monolayer and so any effect on p-glycoprotein (either activation or inhibition) is unlikely, due to the lack of intestinal bacterial enzymes in the calcein-accumulation assay.

5.1.3.4 Genistein

The structure of genistein is shown in Figure 5.1 above. Genistein is a non-citrus flavonoid found in soybeans (~4.6 μg/g soybean) [Fukutake *et al.*, 1996]. It was found in soybean products such as soy powder, soy milk, tofu, bean paste and soy sauce.

Genistein has been found to be a potent inhibitor of tyrosine-specific protein kinase in the epidermal growth factor receptor in the human epidermoid cell line A431 [Akiyama *et al.*, 1987]. In contrast, it had no effect on the enzyme activities of serine- and threonine-specific protein kinases. It was concluded that genistein was not just an ATP analogue but could distinguish between the catalytic sites of a number of enzymes.

There have been a number of studies on the effect of Genistein on p-glycoprotein and MRP. Genistein reduced the accumulation of adriamycin (to 68% control) in HCT-15 colon cells [Critchfield *et al.*, 1994]. It was concluded that genistein upregulates the activity of p-glycoprotein. Genistein was shown to bind to the hexahistidine-tagged C-terminal nucleotide-binding domain (H_6-NBD2) from mouse p-glycoprotein with an IC_{50} of 37 μM [Conseil *et al.*, 1998]. It was concluded that it bound but not as strongly as other compounds.

The presence of genistein (1 μM) caused an ~190% increase in alkaline phosphatase activity in rat hepatocytes [Calhau *et al.*, 2000]. This was a similar effect to known inhibitors of p-glycoprotein (*e.g.* verapamil and cyclosporin A). It was suggested that

genistein might inhibit p-glycoprotein by increasing the dephosphorylation of the transporter through alkaline phosphatase.

Genistein also competitively inhibited daunorubicin transport in the human small cell lung carcinoma cell line GLC4/ADR that was known to overexpress MRP [Versantvoort *et al.*, 1994]. Daunorubicin transport was ATP-dependent and genistein caused a concentration-dependent but modest decrease in ATP levels, resulting in inhibited transport. It was concluded that a modest depletion of cellular energy by genistein inhibited the transport of daunorubicin in MRP-expressing cells.

However, the exposure of the human breast cancer cell line MCF-7 and its p-glycoprotein and MRP expressing sublines to genistein (200 μM) resulted in an increased intracellular accumulation of rhodamine-123 and daunorubicin [Castro & Altenberg, 1997]. It was concluded that genistein inhibits both p-glycoprotein and MRP.

In summary, genistein is an ATP-analogue and is known to affect a number of cellular enzymes. Various studies have found a range of effects of genistein on p-glycoprotein and an inhibitory effect on MRP. Genistein has an indirect effect on the two transports as it affects the phosporylation state of a number of important enzymes and thus affects the cellular environment.

5.1.3.5 Kaempferol

The fruits of *Ginkgo biloba* have been used for medicinal purposes in China for centuries. A leaf extract of this plant has been shown to produce *in vitro* inhibition of rat brain monoamine oxidase (MAO)-A and –B activity [Sloley *et al.*, 2000]. The extract was chromatographed on a reverse-phase HPLC system and two of the components isolated were shown to be MAO inhibitors. These were identified as kaempferol and isorhamnetin. The structure of kaempferol is shown in Figure 5.1 above.

Kaempferol was shown to protect against N-methyl-D-aspartate-induced noradrenergic neuronal toxicity and both kaempferol and *Ginkgo biloba* extract were found to be antioxidants in a lipid-peroxide assay. Although unconjugated kaempferol makes up a small amount (0.6%) of *Ginkgo biloba* extract, this aglycone can arise through the hydrolysis of the flavonol glycosides that are also found in the extract [Pietta *et al.*, 1991].

The extent of DNA damage and lipid peroxidation induced by kaempferol was studied under aerobic conditions in isolated rat-liver nuclei [Sahu & Gray, 1994]. Kaempferol caused a concentration-dependent decrease in double-stranded DNA content concurrent with an increase in lipid peroxidation. Both iron(III) and copper(II) stimulated those reactions probably due to redox reactions with the polyphenolic kaempferol. It was concluded that reactive oxygen species were produced by the kaempferol. A later study [Phang *et al.*, 1994] found that exposing p-glycoprotein expressing MCF-7 cells to N-acetyl-cysteine (a known antioxidant) decreased the peroxide stimulatory effect of kaempferol suggesting the presence of a redox reaction. Therefore, kaempferol has the potential to act as both pro- and anti-oxidants, depending on its redox state.

The p-glycoprotein-mediated efflux of 7,12-dimethylbenz(a)-anthracene in human breast cancer MCF-7 cells was increased by the presence of kaempferol, galangin or quercetin [Phang *et al.*, 1993]. This increase was concentration-dependent and reversed in the presence of a p-glycoprotein inhibitor, either verapamil or quinine. Kaempferol (along with quercetin and galangin) significantly reduced the accumulation of adriamycin (to 42% control) in HCT-15 colon cells [Critchfield *et al.*, 1994]. This effect was abolished in the presence of verapamil.

Kaempferol has been shown to bind to the hexahistidine-tagged C-terminal nucleotide-binding domain (H_6-NBD2) from mouse p-glycoprotein with an IC_{50} of 6.7 μM [Conseil *et al.*, 1998]. It was concluded that it binds strongly to the domain. A derivative of kaempferol, kaempferide, completes with ATP and steroids to bind to the domain. It was concluded that kaempferide (and probably other flavonoids) bind to both the ATP binding site and steroid interacting domain in p-glycoprotein. Flavonoids would therefore affect proteins which bind ATP such as mitochondrial ATPase, Na+/K+ and Ca+ plasma membrane ATPases, protein kinase A and C, tyrosine protein kinase and topoisomerase II.

From these studies it can be concluded that kaempferol stimulates p-glycoprotein however in another study, the presence of kaempferol (100 μM) caused an ~490% increase in alkaline phosphatase activity in rat hepatocytes [Calhau *et al.*, 2000]. This was a similar effect to other known p-glycoprotein inhibitors (*e.g.* verapamil and cyclosporin A). It was suggested that kaempferol might inhibit p-glycoprotein by increasing p-glycoprotein dephosphorylation through alkaline phosphatase.

In summary, kaempferol is a monoamine oxidase inhibitor that has both pro-and antioxidant properties. A number of studies have found kaempferol to have an activating effect of p-glycoprotein although it may indirectly also inhibit p-glycoprotein.

5.3.1.6 Rutin

The structure of rutin is shown in Figure 5.1 above. Rutin is primarily found in buckwheat leaves (23.4 g/kg leaves) and buckwheat seeds (0.2 g/kg seeds) but not other cereals such as oats and barley (<0.1 mg/kg dry mass) [Holasova *et al.*, 2002].

As stated in more detail above, the effect of naringin, rutin and cetechin on xanthine oxidase activity, lipid peroxidation, DNA cleavage and their free radical scavenging capacity was investigated *in vitro* and using rat liver microsomes where necessary [Russo *et al.*, 2000]. It was concluded that naringin, rutin and cetechin all have antioxidant properties.

Rutin has been shown to only very weakly bind to the hexahistidine-tagged C-terminal nucleotide-binding domain (H_6-NBD2) from mouse p-glycoprotein [Conseil *et al.*, 1998]. Rutin also reduced the accumulation of adriamycin (to 64% control) in HCT-15 colon cells [Critchfield *et al.*, 1994] but had no effect on the efflux of adriamycin in MCF7 adriamycin-resistant human breast cells [Scambia *et al.*, 1994].

In summary, the antioxidant rutin weakly binds to and weakly up-regulates the activity of p-glycoprotein.

5.1.4 Furanocoumarin derivatives

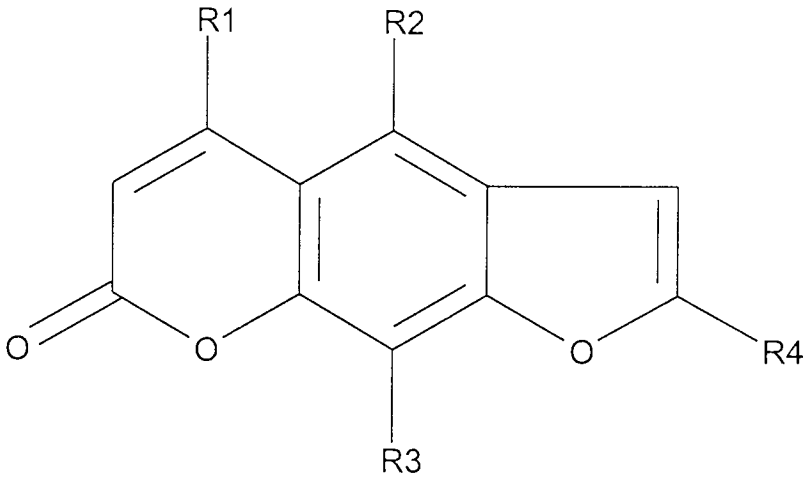
Furanocoumarins are widely distributed in the plant kingdom. The phototoxic constituents include the simple linear furanocoumarins- psoralen, bergapten (5-methoxypsoralen) and xanthotoxin (8-methoxypsoralen). The molecules exert their toxicity through the formation of cross-links between nucleotides. Contact dermatitis following skin contact with furanocoumarin-containing plants (such as celery and citrus) in the presence of bright sunlight is a well-documented phenomenon (*e.g.* "celery picker's itch" and "bartender's itch" from lime oil) however the risks after oral ingestion are very limited [Schlatter *et al.*, 1991].

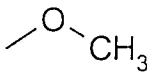
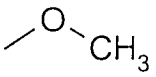
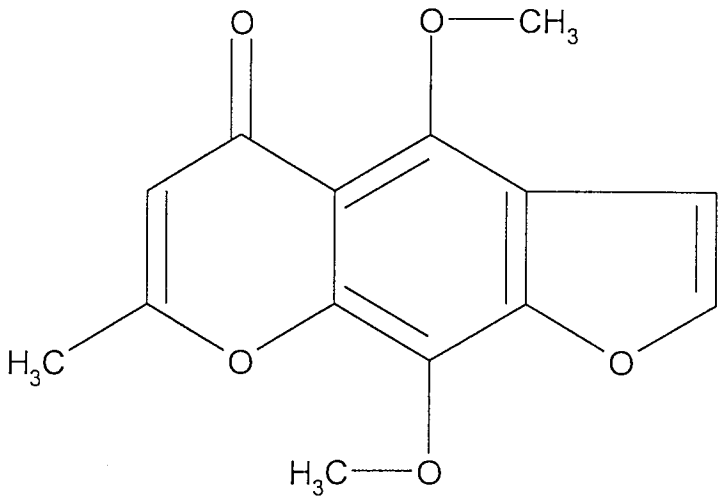
Psoriasis and vitiligo are treated using PUVA therapy (Psoralen and UVA radiation). The furanocoumarins are ingested at high levels (1.2 mg/kg for bergapten) and followed 2-3 hours later by exposure to long-wavelength (320-380 nm) UV radiation. Results are generally obtained after 20 sessions [Bethea *et al.*, 1999].

The two major furanocoumarins in vegetables are bergapten (5-methoxypsoralen) and xanthotoxin (8-methoxypsoralen). The phototoxicity of the two compounds was studied in humans by examining the results of exposure to ultraviolet radiation after consumption of the compounds [Schlatter *et al.*, 1991]. A strong and persistent erythema (skin inflammation and redness) was induced in three of the four subjects who consumed the furanocoumarins solution (15 mg of each compound). The blood concentration levels ranged between 17 and 70 ng/ml for bergapten and between 14 and 114 ng/ml for xanthotoxin before exposure to the light. Two of the subjects consumed a weaker solution (10 mg of each compound). The blood concentrations for both drugs ranged between 12-15 ng/ml. Exposure to ultraviolet light induced pigmentation in one subject and a mild erythema in the other. In the final experiment, celery roots (300 g containing 28.2 μg furanocoumarin/g root) were ingested by the subjects, resulting in blood levels below 2 ng/ml, but exposure to the light caused no skin reactions. It was concluded that the phototoxic threshold for bergapten and xanthotoxin is of the order of 10 mg (blood levels of approximately 10-15 ng/ml) but this level is not reached after consumption of celery roots and other vegetables under a normal dietary habit.

Two other compounds have been used in PUVA therapy: trioxsalen and khellin. Trioxsalen is a pigmenting photosensitizing agent obtained from several plants, mainly the herb *psoralea corylifolia*. It is administered either topically or orally in conjunction with ultraviolet light in the treatment of vitiligo (a skin disease consisting of the development of smooth, milk-white spots upon various parts of the body) [Chuan *et al.*, 1999]. Khellin is also used in the treatment of vitiligo in conjunction with ultraviolet light [Hofer *et al.*, 2001] but it is also a vasodilator [Ubeda & Villar, 1989] that has bronchodilatory properties. It has been employed in the treatment of angina pectis and asthma. The structures of four furanocoumarin derivatives (5-methoxypsoralen, 8-methoxypsoralen, trioxsalen and khellin) are shown in Figure 5.2 below.

Figure 5.2 The structure of the linear furanocoumarin skeleton and some derivatives



Name	R1	R2	R3	R4
5-methoxypsoralen	—H		—H	—H
8-methoxypsoralen	—H	—H		—H
Trioxsalen	$—CH_3$	—H	$—CH_3$	$—CH_3$
Khellin				

Extracts of grapefruit juice were tested *in vitro* for the ability to inhibit CYP3A4 in an attempt to identify active components. Two furanocoumarin derivatives were proposed, 6', 7'-dihydroxybergamottin [Schmiedlin-Ren *et al.*, 1997] and bergapten (5-methoxypsoralen) [Ho *et al.*, 1998]. Both are potent inhibitors of CYP3A4 with very low

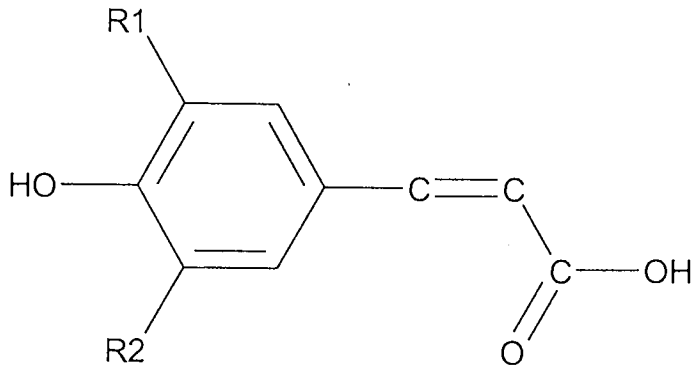
IC50s meaning small variations in concentration result in large variations in inhibitory activity. Bergapten was found in grapefruit juice at low concentrations (~1 mg/L) but this varied between fruit and due to the method of squeezing (by hand, by squeezer or by blender) [Ho *et al.*, 2000]. It was concluded that these variations accounted for the varied results seen during *in vivo* studies of the effect of grapefruit juice. However, an *in vivo* study of the effect of grapefruit juice extracts on felodipine pharmacokinetics [Bailey *et al.*, 1998b] found no correlation between inhibitory activity and the concentration of naringin or 6', 7'-dihydroxybergamottin in the extracts. It was concluded that neither compound was the major active ingredient in grapefruit juice. Another study found that bergamottin is the likely major active component in commercial grapefruit juice, while 6', 7'-dihydroxybergamottin and naringin may be more important in freshly squeezed juice where their concentrations are higher [Bailey *et al.*, 2000].

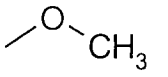
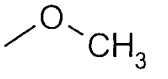
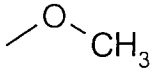
5.1.5 Hydroxycinnamic acids

The health benefits of the polyphenolic flavonoids lead to investigations into the health benefits of other polyphenols. These included the esters, glycosides and amides of the hydroxycinnamic acids. The structures of the four acids investigated in this study are shown in Figure 5.3 below.

Ferulic acid is a ubiquitous plant constituent that arises from the metabolism of phenylalanine and tyrosine [Graf, 1992]. Ferulic acid has been found in coffee, citrus juices, sugar beet fibres and cereal brans [Clifford, 1999]. It readily forms a resonance-stabilised phenoxy radical that can terminate free radical chain reactions [Graf, 1992]. Therefore it is a potent antioxidant. Ferulic acid is thought to be the active ingredient in gamma-oryzanol (also known as rice bran oil), which has been found to lower cholesterol and triglyceride levels [Rukmini & Raghuram, 1991]. Gamma-oryzanol supplements have been claimed to be a natural alternative to anabolic steroids through increasing muscle size and strength, reducing fatigue, protect against cancer, and lower cholesterol, although some of the claims were not supported by published research [Grunewald & Bailey, 1993]. A study of the effect of gamma-oryzanol supplements during resistance training in 22 college-aged males identified the positive effects of training but no significant effects of the supplement. Ferulic acid is marketed as a supplement with many beneficial effects but only the antioxidant properties are supported in the literature.

Figure 5.3 The structure of the hydroxycinnamic skeleton and some derivatives



Name	R1	R2
p-Coumaric acid	—H	—H
Caffeic acid	—H	—OH
Ferulic acid		—H
Sinapinic acid		

Of the other hydroxycinnamic acids, p-coumaric acid has been found in spinach, sugar beet fibre, and cereal brans [Clifford, 1999], caffeic acid has been found in coffee, blueberries, apples and ciders [Clifford, 1999] and sinapinic acid has been found in broccoli, kale, other leafy brassica and citrus juices [Clifford, 1999]. Brown rice and bran contains a number of phenols including caffeic acid, ferulic acid, sinapinic acid and coumaric acid [Hudson *et al.*, 2000]. The hydroxycinnamic acids are found at much lower levels in white rice rather than brown rice therefore worldwide dietary variations may have significant effects. Among citrus peels, the highest concentration of hydroxycinnamic acids occurred in orange (*Citrus sinensis*) followed by tangerine (*Citrus reticulata*), grapefruit (*Citrus paradisi*), and lemon (*Citrus limon*) [Manthey & Grohmann, 2001].

The UK population can be divided into several categories depending on their consumption of coffee, bran and citrus fruits and juices. Those who consume a large number of those

products will easily ingest nearly 1 g of the hydroxycinnamic acids [Clifford, 1999]. Those who don't consume these products and other fruit and vegetables will struggle to consume 25 mg [Clifford, 1999]. There is a high level of variation in the intake of hydroxycinnamic acids and therefore any significant clinical effects and interactions are hard to predict.

The hydroxycinnamic acids have a number of properties. Caffeic acid has been found to have antihyperglycemic properties through the stimulation of α-adrenoreceptors [Cheng & Liu, 2000]. Caffeic acid, coumaric acid and ferulic acid have antimutagenic activities as they inhibited the mutagenic effects of some compounds in *Salmonella typhimurium* [Yamada & Tomita, 1996].

Nitrosamines were discovered more than 100 years ago, but it wasn't until the 1950s that they were found to be highly carcinogenic. They are formed when nitrate salts are reduced to nitrites in the stomach and the nitrites combine with secondary amines to produce the nitrosamines. The effect of a number of compounds on the formation of tobacco-specific N-nitrosamines was investigated [Rundlof *et al.*, 2000]. Caffeic and ferulic acids were potent inhibitors of the reaction of nornicotine and nitrite, with p-coumaric acid having less potency. The same pattern of potency was seen in the inhibition of nitrosamine formation in tobacco midrib and lamina systems. The three compounds are nitrite scavengers, with caffeic and ferulic acid as the most potent.

A radical scavenging test using 1,1-diphenyl-2-picrylhydazyl was used to investigate the hydrogen-donating potency of the hydroxycinnamic acids [Kikuzaki *et al.*, 2002]. Activity decreased in the order: caffeic acid (49.6%) > sinapinic acid (33.2%) > ferulic acid (26.3%) > *p*-coumaric acid (6.0%). Other experiments, including investigating the inhibitory effect of compounds on autoxidation of methyl linoleate in bulk phase, found a similar pattern.

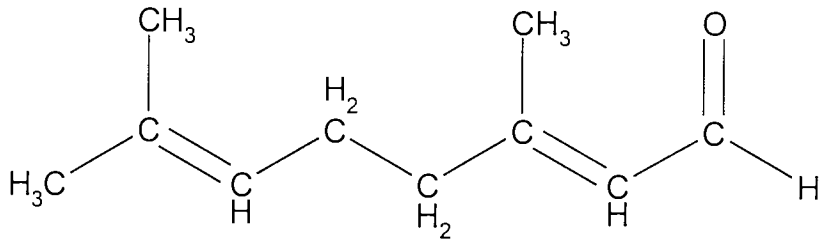
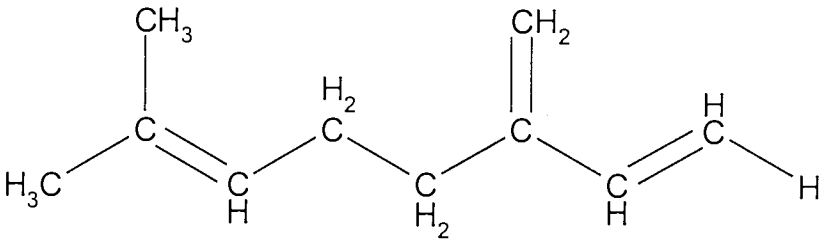
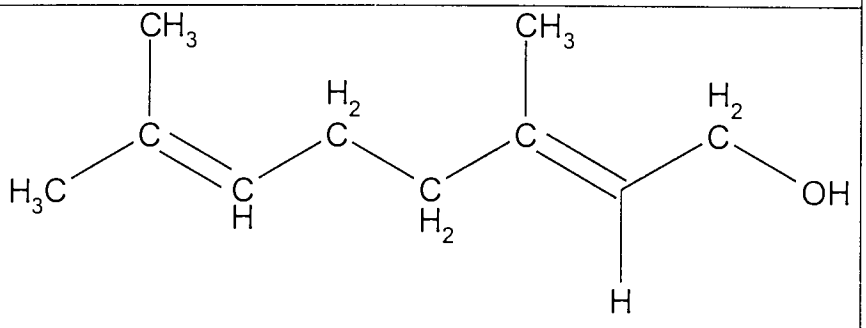
Radical scavenging potency appeared related to the presence of an oxygen atom on the phenolic ring. The most potent was caffeic acid (with a hydroxyl group), then sinapinic acid (with two methoxy groups), then ferulic acid (with one methoxy group), and finally p-coumaric acid (with no extra groups).

In summary, the hydroxycinnamic acids are ubiquitous polyphenolic compounds. They have been found to be potent radical scavengers but few other properties have been identified.

5.1.6 Natural linear compounds

The structures of some of the tested linear natural compounds found in bergamot and grapefruit oil are shown in Figure 5.4 below.

Figure 5.4 The structures of some of the linear natural compounds found in bergamot and grapefruit oil

Name	Structure
Citral	
Myrcene	
Geraniol	

Citral (3,7-dimethyl-2,6-octadienal) is the principal component of lemongrass oil. The short-term effects of citral on the livers of Long Evans hooded and Wistar albino rats were investigated [Jackson *et al.*, 1987]. Treatment with citral led to liver enlargement (hepatomegaly), an altered distribution of lipid and glycogen in the liver and peroxisome

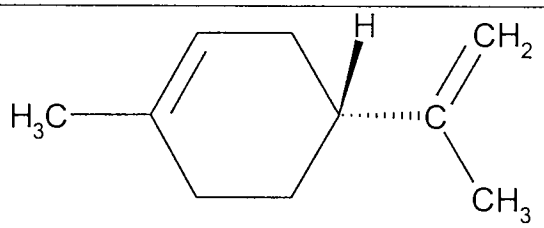
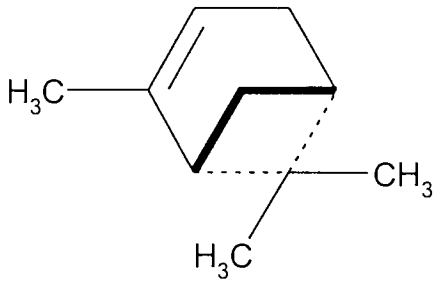
proliferation (due to enzyme inductions). A later study on male Wistar rats [Roffey *et al.*, 1990] found that administration of citral (1.5 g citral/kg body weight/day for 5 days) caused the induction of peroxisome enzymes and microsomal CYP4A1.

In contrast to citral, there has very little research performed on myrcene and geraniol. Myrcene is a monoterpene found in the essential oils of various plants, including lemongrass (Cymbopogon citratus) and is widely used in Brazilian folk medicine. Geraniol is a primary alcohol, and constitutes the chief part of oil of rose and palmarosa oil. It is also found in geranium, citronella, lemon and many other oils.

5.1.7 Natural cyclic compounds

The structures of some of the cyclic natural compounds found in bergamot and grapefruit oil tested are shown in Figure 5.5 below.

Figure 5.5 The structures of some of the cyclic natural compounds found in bergamot and grapefruit oil

Name	Structure
(R)-(+)-Limonene	
(-)-α-Pinene	

Limonene is found in citrus fruits, mint, myristica, caraway, thyme, cardamon, coriander and many other oils. The active isomer is (+)-limonene but also known as d-limonene. Extracted d-limonene is used as a lemon fragrance in soaps and creams and as a flavouring agent in foods, drinks and chewing gum.

It is rapidly absorbed from the gastrointestinal tract and metabolised in man into two glycols [Watabe *et al.*, 1981]. The major metabolite is d-limonene 8,9-glycol and the minor metabolite is d-limonene 1,2-glycol. Dosing male Wistar rats with a single dose of d-limonene caused no effects on liver triglycerides, microsomal proteins, cytochrome b5 or other drug metabolising enzymes [Ariyoshi *et al.*, 1975]. Longer term dosing resulted in a slight increase in liver weight, decreased liver and serum cholesterol, and increased levels of cytochromes P450 and b5.

Mammary tumours induced by 7,12-dimethylbenz[a]anthracene (DMBA) in female rats were regressed by the consumption of a diet containing 10% d-limonene [Elegbede *et al.*, 1986]. A later study found that rats fed a diet of 5% limonene had elevated hepatic glutathione-S-transferase activity and elevated hepatic uridine diphosphoglucuronosyl transferase activity. It was proposed that a majority of the anticarcinogenic activity of limonene is mediated through the induction of the two hepatic enzymes.

Mice given 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) develop tumours in the lungs and forestomach. The consumption of d-limonene, orange oil or lemon oil one hour prior to dosing with NNK, inhibited pulmonary adenoma formation and the occurrence of forestomach tumours [Wattenberg & Coccia, 1991]. Therefore, it can be seen that d-limonene has a range of anticarcinogenic properties.

In contrast to limonene, there have been few investigations into the chemical properties of α-pinene. α-Pinene is a 10-carbon monoterpene biosynthesized from two isoprene (2-methyl- 1,3-butadiene) units (Figure 5.5). It is a major component of wood turpentine which can be obtained from the resinous sap of pine trees (Pinus) by steam distillation.

5.1.8 Aim of chapter

The aim of this chapter is to investigate the effect of a range of natural compounds on the activity of p-glycoprotein. The first objective is to identify the effect of grapefruit juice on the modified calcein assay. The effects of fruit juice (grapefruit, lemon and orange) formulations (pH 3, pH 7, rehydrated) and oil emulsions (grapefruit and bergamot) will be investigated to define the significance of any effects. Compounds that are known to be CYP3A4 inhibitors (flavones and furanocoumarins) or antioxidants (hydroxycinnamic

acids) will be tested, as they are likely to be inhibitors of p-glycoprotein as well. Finally, the natural linear and cyclic compounds will be tested to investigate if the oxidation state and three-dimensional arrangements of the molecule affect the ability to inhibit p-glycoprotein.

5.2 Materials and methods

5.2.1 Preparation of grapefruit juice formulations

5.2.1.1 Freshly squeezed grapefruit juice

Two normal sized grapefruit (*Citrus paradisi*) were purchased from a supermarket, cut in half and both halves squeezed using an ordinary juicer. The two volumes of juice were then centrifuged at 1000 rpm for 5 minutes and the supernatants collected. These juices had a pH of 2.74 and were referred to as Grapefruit Juice (pH 3) samples 1 and 2.

5.2.1.2 Buffered grapefruit juice

Two sample of the freshly squeezed juice (25ml Grapefruit Juice (pH 3)) were separately mixed with HEPES buffer (0.15 g to make two 25 mM solutions) and the pH adjusted to 7 by the addition of sodium hydroxide (0.25 M, dropwise). These juices were referred to as Grapefruit Juice (pH 7) samples 1 and 2.

5.2.1.3 Rehydrated freeze-dried grapefruit puree

Freeze-dried grapefruit puree powder was obtained from Confoco (UK) Ltd. (Surrey, UK). This puree was reconstituted with PBS containing 5 mM glucose, at a concentration of 30 mg/ml (0.75 g in 25 ml), buffered to pH 7 and used as a test solution.

5.2.2 Preparation of other test solutions

5.2.2.1 Fruit juices

An orange (*Citrus sinensis*) and a lemon (*Citrus limon*) (obtained from a supermarket) were squeezed as in Section 5.2.1.1 and buffered as in Section 5.2.1.2 to produce the solutions tested. The buffered solutions were also diluted to 50% with PBS containing 5 mM glucose to identify any dose effect.

5.2.2.2 Fruit oils

The cold pressed oil from grapefruits was bought from an essential oil shop (Zen, Birmingham, UK). The oils were shaken with PBS containing 5 mM glucose to make 1% and 10% emulsions. The emulsions were thoroughly mixed before being used in the assay.

5.2.2.3 Other compounds

All other compounds used were supplied by Sigma (UK) Ltd. The compounds were dissolved in PBS containing 5 mM glucose with the addition of DMSO or ethanol as a co-solvent where needed. The compounds were stored as 1 mM solutions in a –20°C freezer until needed. The solutions were then diluted to make 25, 50 and 100 μM solutions with the co-solvent at no more than 5% (shown to have no significant effect in Figure 4.11).

5.2.3 Effect of compounds on calcein-AM assay

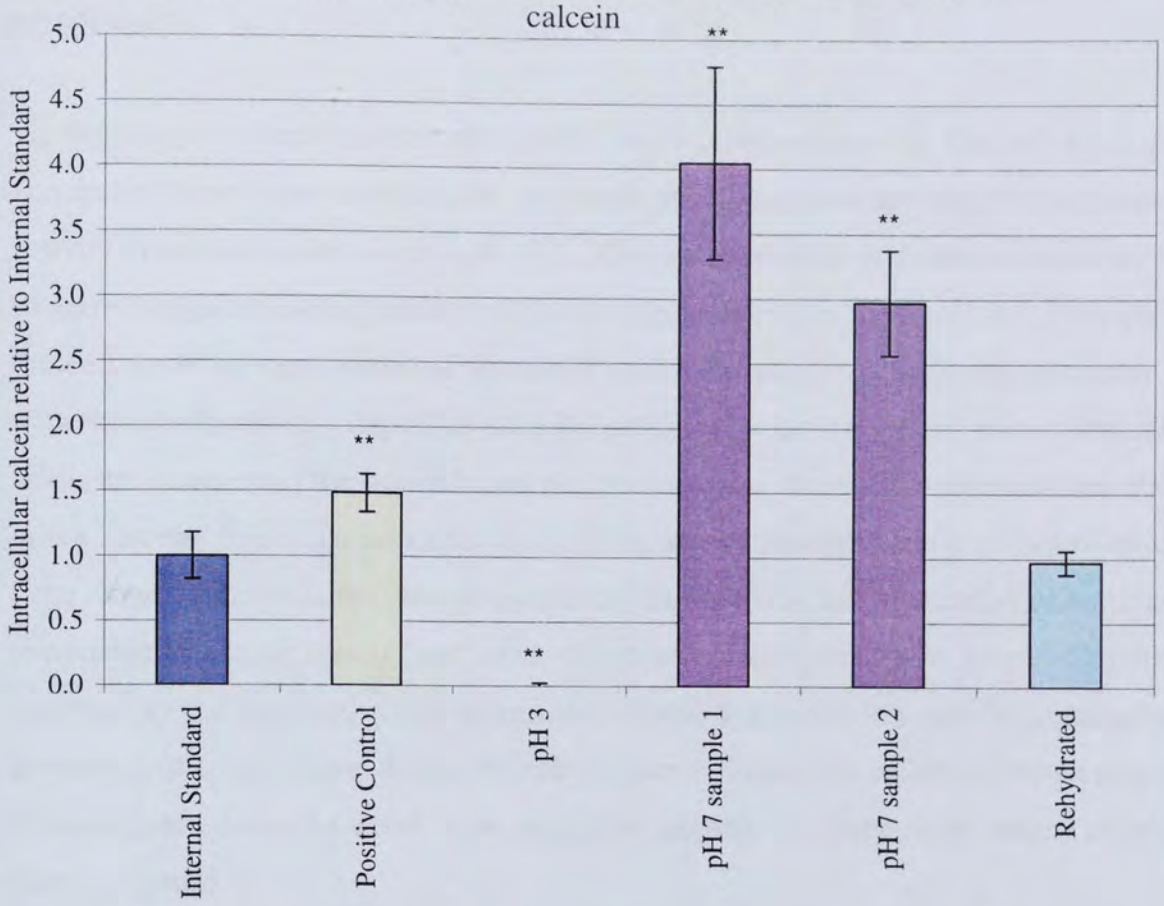
See Section 4.2.7 for a complete description. In brief, caco-2 cells were split, counted and resuspended in PBS containing 5 mM glucose. The cell suspension was aliquoted into a 96-well plate and the test solution added. The plate was then incubated at 37 °C for 30 minutes (pre-ester incubation). The calcein-AM was then added and the plate incubated at 37 °C for 30 minutes (post-ester incubation). Cobalt chloride was then added to each well and the fluorescence read immediately (excitation at 485 nm, emission at 538 nm). The plate was shake to resuspend the cells before the fluorescence was read. The 96-well plate was set up to allow the measurement of the effect of six test solutions, the positive control (100 μM cyclosporin A) and the internal standard (PBS containing 5 mM glucose), with four wells for each condition.

5.3 Results and discussion

5.3.1 Effect of different grapefruit juice formulations

Figure 5.6 below shows the effect on intracellular calcein of the internal standard, positive control (100 μM cyclosporin A) and various formulations of grapefruit juice: Grapefruit Juice (pH 3) sample 1, Grapefruit Juice (pH 7) samples 1 and 2 and rehydrated freeze-dried grapefruit puree.

Figure 5.6 Effect of grapefruit juice formulations on intracellular calcein



Data are mean and standard deviation of four wells expressed as intracellular calcein relative to the Internal Standard. The data were collected from multiple experiments therefore only one of the positive controls is presented to illustrate the size of the effects of the grapefruit juice formulations. An ANOVA analysis and Dunnett's post-test were used to identify conditions that had significantly different intracellular calcein levels to the internal standard. ** Denotes a very significant ($p<0.01$) difference from

The addition of Grapefruit Juice (pH 3) resulted a significant decrease in the intracellular calcein concentration. This strongly acidic solution is probably toxic for the cells, which would be unable to do anything to the calcein-AM ester. The end result is that calcein did not accumulate inside the cells. Other solutions of pH 3 will need to be tested to confirm that the effect is due to the pH and not the activity of a grapefruit juice component at pH 3. Methods such as cell microscopy or the MTT and Trypan blue assays could be used to measure cell viability.

The effect of Grapefruit Juice pH 7 is clearly significant. There is at least a three-fold increase in intracellular calcein relative to the internal standard and a two-fold increase relative to the positive control. Although both pH 7 solutions from two different

grapefruits caused a significant ($p<0.01$) increase in intracellular calcein, the sizes of the increases were significantly ($p<0.05$) different. This difference may be due to the interfruit variation in concentrations of the compounds which together significantly inhibit p-glycoprotein.

As the assay has been shown to be specific for p-glycoprotein (see Chapter 4), it can be concluded from these results that grapefruit juice components inhibit p-glycoprotein activity in an environment with a pH of 7. The clinical studies that demonstrated the effect of grapefruit juice on drug pharmacokinetics were reviewed in Section 1.8.3. The question remains over the significance of the assay results for people consuming grapefruit juice with their medication. Grapefruit juice is consumed without a buffer, passes through the acidic environment of the stomach, and into the intestine. Here the presence of bicarbonate in the secreted bile acids raises the local pH to approximately 7. It is at this point that a large range of compounds is absorbed into the blood stream *via* endothelial cells. It seems reasonable to expect that at least some of the active components in grapefruit juice are absorbed by the cells and could conceivably inhibit p-glycoprotein activity increasing the absorption of co-consumed drugs. Further studies will show the effect of known grapefruit juice components on the assay in an attempt to identify the compounds whose effects are seen in Figure 5.6.

There was no significant ($p>0.05$) effect on intracellular calcein in the presence of rehydrated freeze-dried grapefruit puree. There are a number of possible reasons for this result. Although the freeze-drying process only removes water and rehydrating only adds water, the processes might damage the quaternary structures of the active ingredients in the grapefruit inactivating them. This effect was identified following the freeze-drying and rehydration of L-asparaginase where the active tetramer dissociated when freeze-dried [Hellman *et al.*, 1983]. Complete reassociation was dependent on the pH of the buffers.

The fact that powdered grapefruit puree (from the whole fruit) was tested might mean that the active ingredients were at a much lower concentration than in grapefruit juice and therefore have no effect. Another possible reason is that the powder was rehydrated with an inappropriate volume of PBS containing 5 mM glucose diluting the effect of the active ingredients.

In summary there are a number of possible theories for the inactivity of the rehydrated grapefruit puree. A number of experiments would need to be performed to identify the correct theory. As the puree undergoes a number of processes, samples could be taken and put through the assay to clearly identify the stage at which it loses its inhibitory activity. The rehydrated puree could be used in a clinical setting as an inactive replacement for grapefruit juice if that was needed. These experiments remain to be performed in the future.

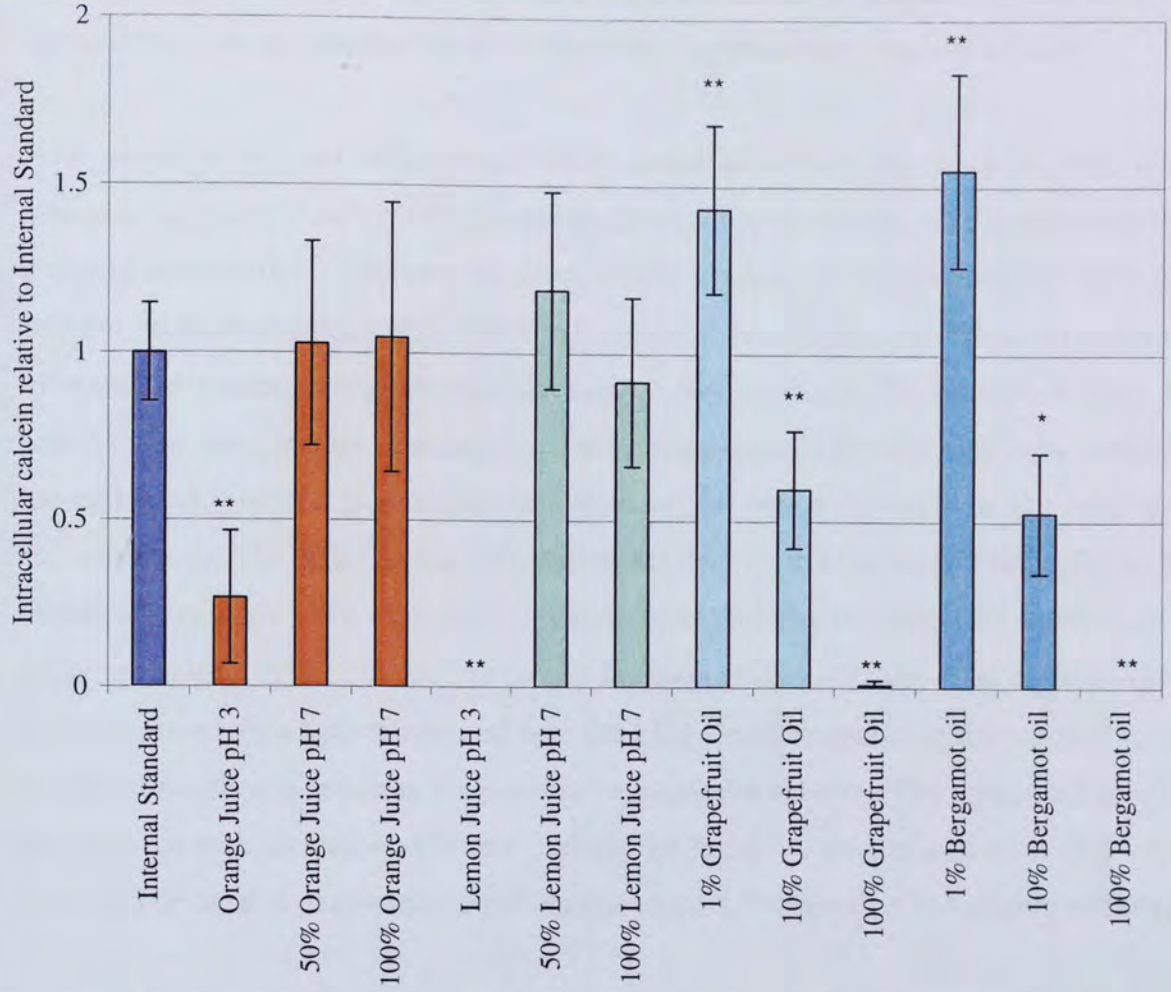
In summary, grapefruit juice (pH 3) is strongly acidic and is toxic to the cells used in the assay. However, buffering the juice to pH 7 results in a significant inhibition of p-glycoprotein activity. This result may be clinically relevant as the acidic contents of the stomach are buffered and the pH is raised as food progresses through the small intestine. Rehydrated grapefruit puree had no significant effect on the assay and a number of possible reasons why have been suggested. A number of constituents of grapefruit juice probably work together to inhibit p-glycoprotein. The activity of other juices and oils should be examined to reduce the number of candidate active compounds in grapefruit juice.

5.3.2 Effect of other juices and oils

Figure 5.7 below shows the effect on the assay of the internal standard and various concentrations of two fruit juices (orange and lemon) and two oils (grapefruit and bergamot).

Both freshly squeezed orange and lemon juice cause a significant ($p<0.01$) decrease in intracellular calcein relative to the internal standard. This is a similar effect to the addition of freshly squeezed grapefruit juice seen in Figure 5.6. The previous conclusion is supported *i.e.* the strongly acidic solutions are unable to do anything to the calcein-AM ester, resulting in the lack of calcein accumulated inside the cells. The variation in intracellular calcein between the juices is probably due to variations in the acidity of the juices affecting the toxicity of the juices. When the juices were buffered to pH 7, no significant ($p>0.05$) effect on intracellular calcein was seen for either juice or at either concentration (50% or 100%). These results contrast with the grapefruit juice results shown in Figure 5.6. It was concluded that orange juice and lemon juice have no significant effect of the activity of p-glycoprotein.

Figure 5.7 Effect on intracellular calcein of two fruit juices (orange and lemon) and two oils (grapefruit and bergamot)



Data are mean and standard deviation of four wells expressed as intracellular calcein relative to the Internal Standard. The data were collected from multiple experiments therefore none of the positive controls are presented. An ANOVA analysis and Dunnett's post-test were used to identify conditions that had significantly different intracellular calcein levels to the internal standard. * Denotes a significant ($p<0.05$) difference from the internal standard. ** Denotes a very significant ($p<0.01$) difference from the internal standard.

Some components of orange juice (the methoxyflavones tangeretin, nobiletin and heptamethoxyflavone) have been shown to inhibit vincristine transport and hence p-glycoprotein activity *in vitro* [Ikegawa *et al.*, 2000]. However it was not demonstrated how clinically relevant the results were. The results presented here show that orange juice has no significant effect on the assay therefore any active components of orange juice must be found at a low enough concentration to have no measurable effect. Components of orange juice that are found in grapefruit juice at a similar concentration can be deemed to not be the active ingredients in grapefruit juice.

Lemon juice has previously been found to inhibit CYP3A4 but with different inhibition kinetics to grapefruit juice [Baltes *et al.*, 2001]. There have been no studies examining the effect of lemon juice on p-glycoprotein. Lemon juice has no significant effect on p-glycoprotein. Components of lemon juice that are found in grapefruit juice at a similar concentration can be deemed to not be the active ingredients in grapefruit juice.

Both grapefruit oil and bergamot oil have similar effects on the assay. A 1% emulsion causes a very significant ($p<0.01$) increase in intracellular calcein, a 10% emulsion causes a significant ($p<0.05$) decrease in intracellular calcein (to approximately 50% of the internal standard result), and a 100% oil causes a very significant ($p<0.01$) decrease in intracellular calcein (to approximately zero). Even though 1% emulsions were being added to the assay instead of solutions, some components of the oils may have entered into the cells and inhibited p-glycoprotein. Both of the oils were toxic to the cells at high concentrations. The effect of the 10% emulsions may be the results of both effects. Some of the components were inhibiting p-glycoprotein but the oil was also interacting with either the calcein-AM or the cell to reduce the intracellular calcein. The calcein-AM may partition more in the hydrophobic oil than the PBS therefore reducing the amount available to enter the cells and reducing the possible intracellular calcein. The hydrophobic oil may also interact with the cell membranes and disrupt them. A control emulsion (*e.g.* paraffin oil) could be used to determine the effects due to the oil rather than the other constituents.

Grapefruit oil is extracted from grapefruit peel and contains some of the constituents of grapefruit juice. Grapefruit juice has been shown to have a significant ($p<0.01$) effect on the assay so it is not surprising to identify the inhibitory effect of grapefruit oil. Grapefruit oil has previously been found to increase doxorubicin accumulation in human MCF-7 cells expressing p-glycoprotein [Abedel-nasser *et al.*, 2000]. The biggest effect was seen with a 1% emulsion.

The name of the group of compounds called bergamottins was derived from the bergamot fruit they were initially found in. Given that many bergamottins have been found to be p-glycoprotein inhibitors, it is reassuring that bergamot oil (1% emulsion) significantly ($p<0.01$) increased intracellular calcein.

In summary, the fruit juices tested (orange and lemon) only had a significant effect on the assay when they were freshly squeezed and therefore very acidic. Juices buffered to pH 7

had no effect on intracellular calcein suggesting that the components of orange and lemon juices are not inhibitors of p-glycoprotein. Both the oils tested (grapefruit and bergamot) were toxic to the cells and abolished calcein accumulation at high concentrations. However, 1% emulsions of both oils significantly increased intracellular calcein, indicating the presence of inhibitors of p-glycoprotein activity. Other oils (*e.g.* olive, soy or corn) should be tested to identify the effects that are due to the oils and not the components in grapefruit and bergamot oils. Cell viability tests would also prove whether the oils are cytotoxic or just interfering with the calcein-AM. Compounds that are components of grapefruit juice and either grapefruit or bergamot oil should be tested, as they are potentially the active ingredients of grapefruit juice. More studies are needed to extract and identify those compounds.

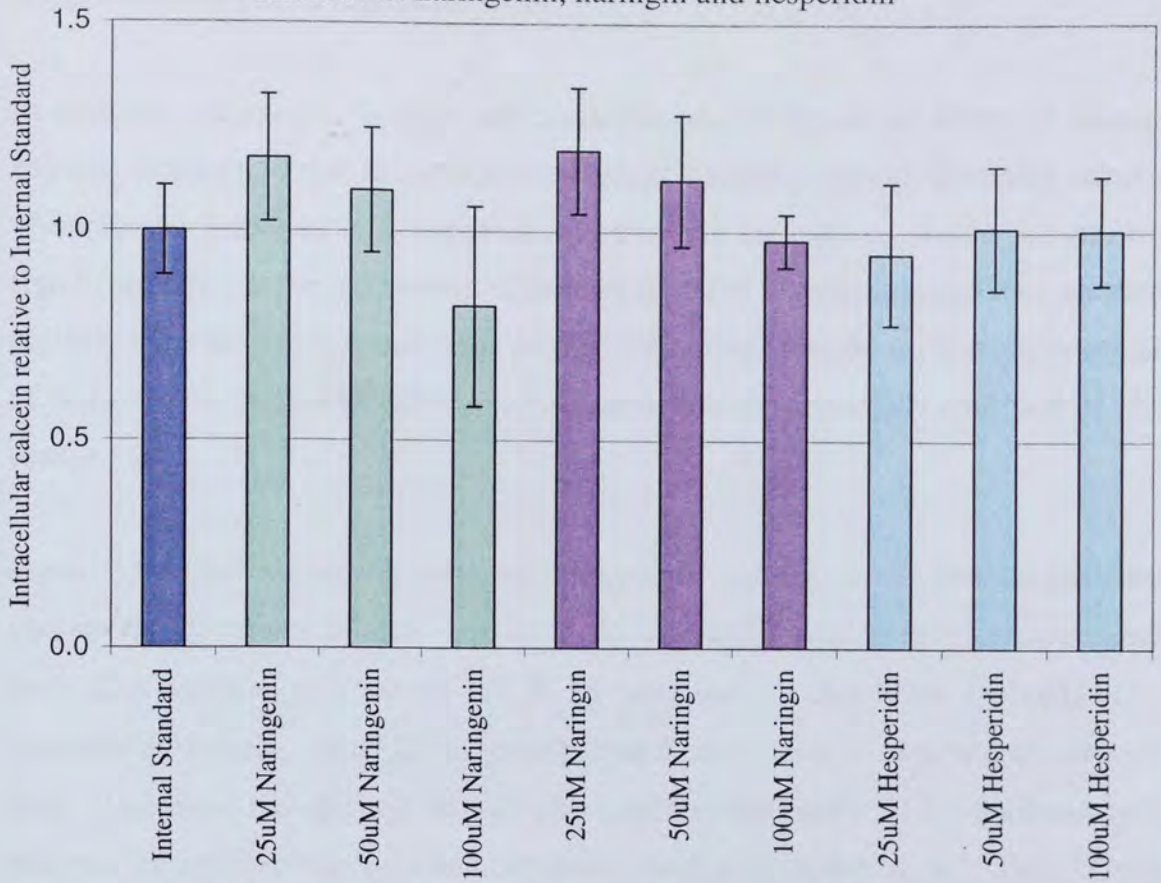
5.3.3 Effect of flavone derivatives

Figure 5.8 below shows the effect on intracellular calcein of the internal standard and various concentrations of three flavone derivatives found in citrus fruits: naringenin, naringin and hesperidin.

The addition of naringenin (25-100 μM) had no significant ($p>0.05$) effect on intracellular calcein Naringenin is found in grapefruit juice [Ho *et al.*, 2000] at approximately 60 μM, a concentration where it would have no significant effect on intracellular calcein. Therefore, the very significant effect of grapefruit juice (pH 7) on intracellular calcein (seen in Figure 5.6) is not due to the presence of naringenin.

The addition of naringin (25-100 μM) had no significant ($p>0.05$) effect on intracellular calcein. Naringin is found in grapefruit juice [Ho *et al.*, 2000] at approximately 200-1200 μM. Although no concentrations in this range were tested, it would be expected that at the higher concentration, naringin would either have no significant effect or cause a significant decrease in intracellular calcein. Therefore, the very significant increasing effect of grapefruit juice (pH 7) on intracellular calcein (seen in Figure 5.6) is unlikely to be due to the presence of naringin. The higher concentration of naringin needs to be tested using the modified assay before conclusions can be made.

Figure 5.8 Effect on intracellular calcein of the citrus flavone derivatives: naringenin, naringin and hesperidin



Data are mean and standard deviation of four wells expressed as intracellular calcein relative to the Internal Standard. The data were collected from multiple experiments therefore none of the positive controls are presented. An ANOVA analysis and Dunnett's post-test were used to show that the conditions were not significantly different to the Internal Standard.

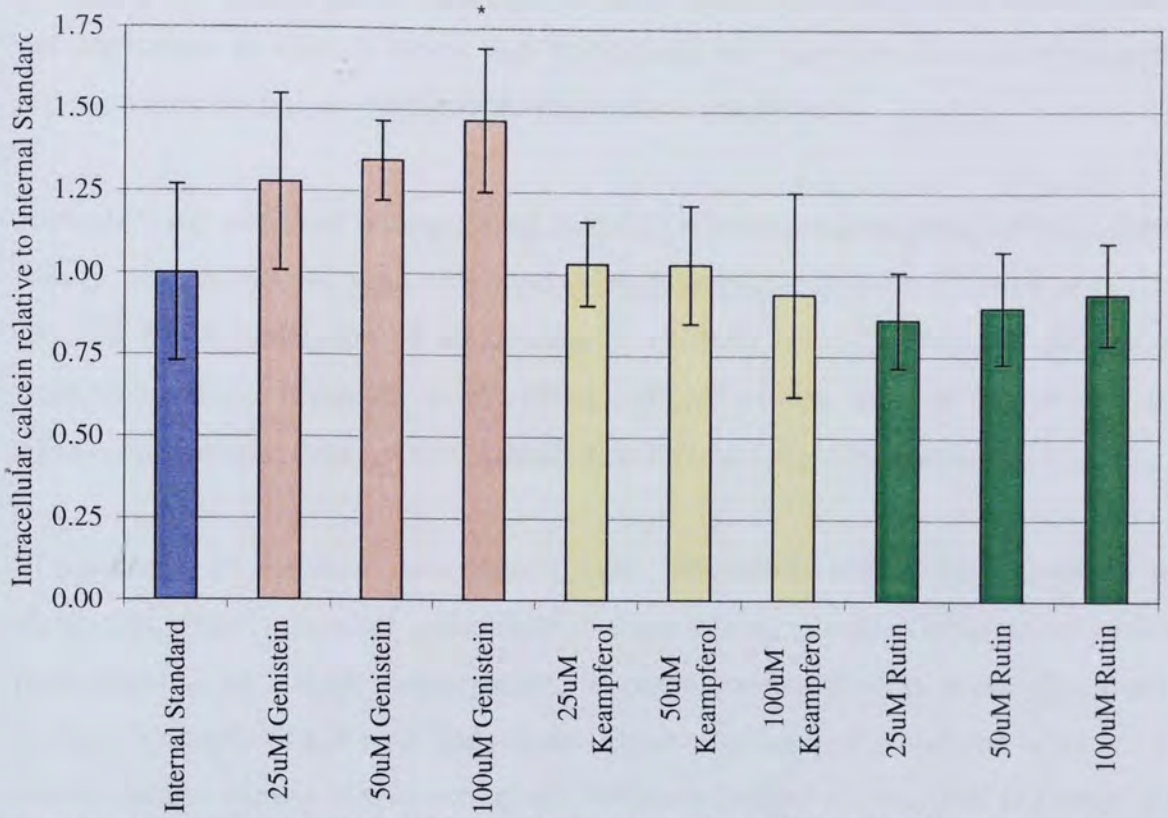
The addition of hesperidin (25-100 μM) had no significant ($p>0.05$) effect on intracellular calcein; no non-significant concentration-dependent effect was seen (unlike in the presence of naringenin and naringin). Previous studies have found that hesperidin either had no effect on p-glycoprotein [Mitsunaga *et al.*, 2000] or a weak activating effect [Critchfield *et al.*, 1994]. Hesperidin is found in grapefruit juice [Ameer *et al.*, 1996] at approximately 15.9 μM (9.72±0.4 mg/L), a concentration not tested in this study. However, as hesperidin did not have a significant effect at 25 μM or higher, it is not expected that it would have a significant effect at 15.9 μM. Therefore, the very significant increasing effect of grapefruit juice (pH 7) on intracellular calcein (seen in Figure 5.6) is unlikely to be due to the presence of hesperidin. Hesperidin is found in orange juice [Ameer *et al.*, 1996] at a concentration of 106 μM, a concentration just outside of the range tested. However, the lack of a significant effect at concentrations up to 100 μM and lack of non-significant dose-dependent effects existing, imply that it is highly unlike that any significant effect

will be seen at this slightly higher concentration. Orange juice (pH 7) had no effect on intracellular calcein (see Figure 5.6) so it is no surprise that a major orange juice component also had no significant effect.

In summary naringenin, naringin and hesperidin had no significant effect on intracellular calcein. Naringenin and naringin may have had a non-significant activating effect on p-glycoprotein. However it is expected that all three compounds would not have had a significant effect at the concentrations they are found in grapefruit juice. They are therefore unlikely to be the active components of grapefruit juice. Hesperidin is a major component of orange juice; its lack of effect was consistent with the previously seen lack of effect of orange juice.

Figure 5.9 below shows the effect on intracellular calcein of the internal standard and various concentrations of three non-citrus flavone derivatives: genistein, kaempferol and rutin. The addition of genistein (25 & 50 μM) had no significant ($p>0.05$) effect on intracellular calcein, although a non-significant concentration-dependent increase was seen. However, the addition of 100 μM genistein did result in a significant ($p<0.05$) increase in intracellular calcein. Previous studies [Conseil *et al.*, 1998, Castro & Altenberg, 1997 & Calhau *et al.*, 2000] have found that genistein binds to and inhibits p-glycoprotein and MRP [Versantvoort *et al.*, 1994 & Castro & Altenberg, 1997] although one study found it had a weak activating effect on p-glycoprotein [Ctitchfield *et al.*, 1994]. The modified assay has been found to be specific for p-glycoprotein, with MRP only playing a minor non-significant role (Chapter 5). The results shown in Figure 5.9 below lead to the conclusion that genistein inhibits the efflux activity of p-glycoprotein, but only at a high concentration. Genistein is therefore a p-glycoprotein inhibitor.

Figure 5.9 Effect on intracellular calcein of three non-citrus flavone derivatives: genistein, kaempferol and rutin



Data are mean and standard deviation of four wells expressed as intracellular calcein relative to the Internal Standard. The data were collected from multiple experiments therefore none of the positive controls are presented. An ANOVA analysis and Dunnett's post-test were used to identify conditions that had significantly different intracellular calcein levels to the internal standard. * Denotes a significant ($p<0.05$) difference from the internal standard.

The clinical relevance of the effect of genistein is unknown. It has been calculated that Japanese men consume approximately 1.5-4 mg genistein/day [Fukutake *et al.*, 1996] but as the volume of food consumed is unknown, the peak concentration of genistein passing through the gastrointestinal tract is also unknown. The high level of consumption is thought to be associated with the lower incidence of, and mortality from, breast, colon and prostate cancer in Japanese men compared to American and Western European men [Fukutake *et al.*, 1996].

The addition of kaempferol (25-100 μM) had no significant ($p>0.05$) effect on intracellular calcein. No significant or non-significant concentration-dependent effect was seen (unlike in the presence of genistein). Previous studies have found that kaempferol generally had an activating effect on p-glycoprotein [Phang *et al.*, 1993 & Critchfield *et al.*, 1994]. No effect was seen in Figure 5.9. Kaempferol is found in extracts of *Ginkgo biloba*, which is

taken as a natural compound for the brain and circulatory system. However, a recent study [Solomon *et al.*, 2002] found that subjects consuming *Ginkgo biloba* over a six-week period did not have a measurable improvement in memory or related cognitive function compared to a control group. Kaempferol might have a systemic effect when the extracts are consumed, however it seems that kaempferol will have no effect on p-glycoprotein although the effect of its metabolite kaempferide is unknown.

Rutin (25-100 μM) had no significant ($p>0.05$) effect on intracellular calcein. Previous studies have found that rutin only weakly binds to p-glycoprotein [Conseil *et al.*, 1998], reduced the accumulation of adriamycin [Critchfield *et al.*, 1994] and had no effect adriamycin efflux [Scambia *et al.*, 1994]. No effect was seen in Figure 5.9. It was concluded that rutin does not significantly affect the activity of p-glycoprotein.

In summary, of the three non-citrus flavone derivatives tested, only genistein had a significant effect (equivalent to the effect of the positive control). Genistein only inhibited p-glycoprotein at a high concentration, a result consistent with previously published studies. Kaempferol and rutin had no significant effect on p-glycoprotein activity. These results are also consistent with previously published studies. Kaempferol is known to have a number of effects on the body and although it does not have a direct effect on p-glycoprotein, its metabolites might.

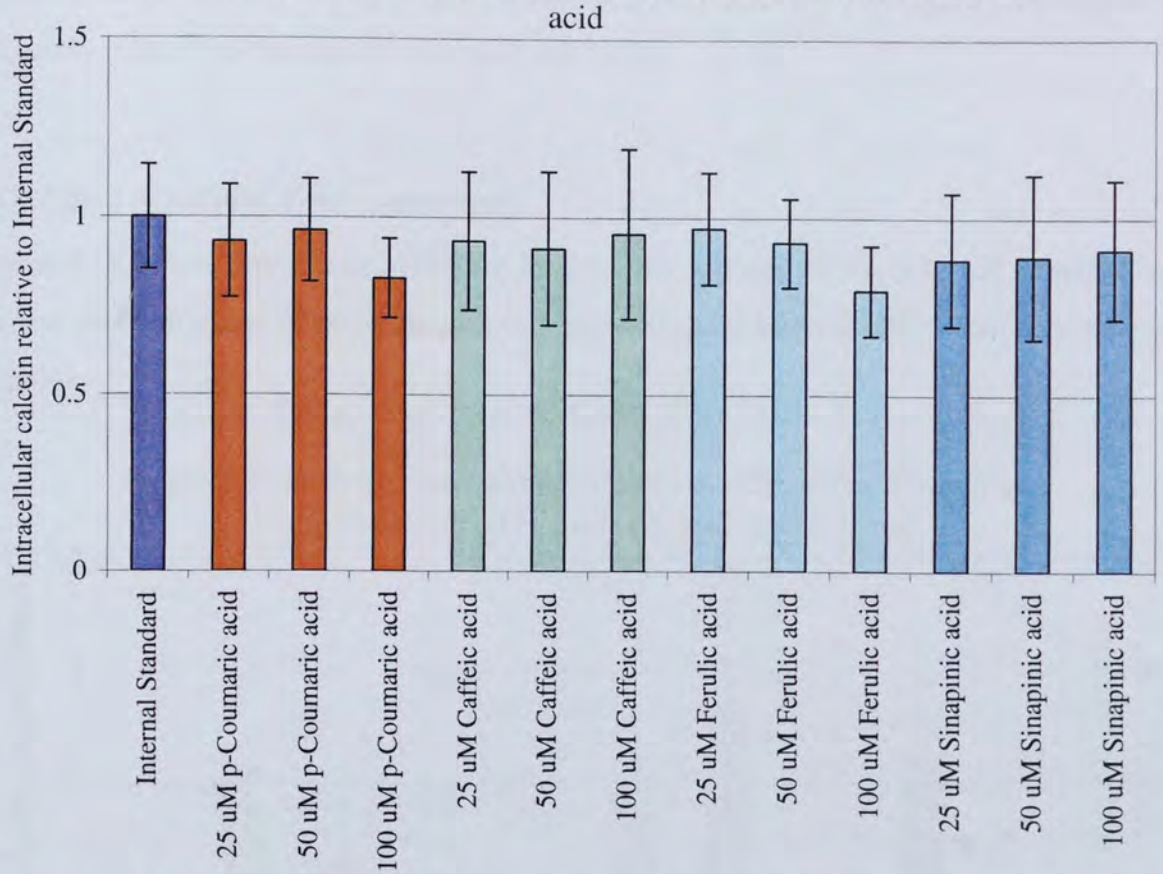
5.3.4 Effect of furanocoumarins

Four furanocoumarins (5-methoxypsoralen, 8-methoxypsoralen, trioxsalen and khellin) were found to be poorly soluble in PBS containing 5% glucose with either DMSO or ethanol as co-solvents (used at concentrations up to 5%, the maximum tested in Figure 4.7). Previous studies [Schlatter *et al.*, 1991] dissolved 15 mg 5-MOP or 8-MOP in 50% alcohol solutions. 5-methoxypsoralen (5-MOP) is found in grapefruit juice at a concentration of 1 mg/L (~4 μM) [Ho *et al.*, 2000] yet it is such a potent inhibitor of CYP3A4 that it is thought to be the major contributor of the effect of grapefruit juice on CYP3A4 [Ho *et al.*, 1998]. The furanocoumarins remain the components of grapefruit juice that are most likely to inhibit p-glycoprotein yet their poor solubility remains a barrier to proving this.

5.3.5 Effect of hydroxycinnamic acids

Figure 5.10 below shows the effect on intracellular calcein of the internal standard and various concentrations of four hydroxycinnamic derivatives: p-coumaric acid, caffeic acid, ferulic acid and sinapinic acid.

Figure 5.10 Effect on intracellular calcein of four hydroxycinnamic derivatives: p-coumaric acid, caffeic acid, ferulic acid and sinapinic acid



Data are mean and standard deviation of four wells expressed as intracellular calcein relative to the Internal Standard. The data were collected from multiple experiments therefore none of the positive controls are presented. An ANOVA analysis and Dunnett's post-test were used to identify conditions that had significantly different intracellular calcein levels to the internal standard.

Ferulic acid and sinapinic acid have been found in citrus juice [Clifford, 1999] making then potential inhibitors of p-glycoprotein. All the hydroxycinnamic acids have the phenolic ring and antioxidant properties [Graf, 1992] that many inhibitors of p-glycoprotein also have. The effect of these acids on p-glycoprotein activity has never been studied. Therefore the four acids were screened for inhibitory activity. However, it is clear from Figure 5.10 above that the hydroxycinnamic acids have no significant effect on the assay and therefore on p-glycoprotein activity. There is no indication that higher concentrations may have a significant effect although that cannot be ruled out. There are

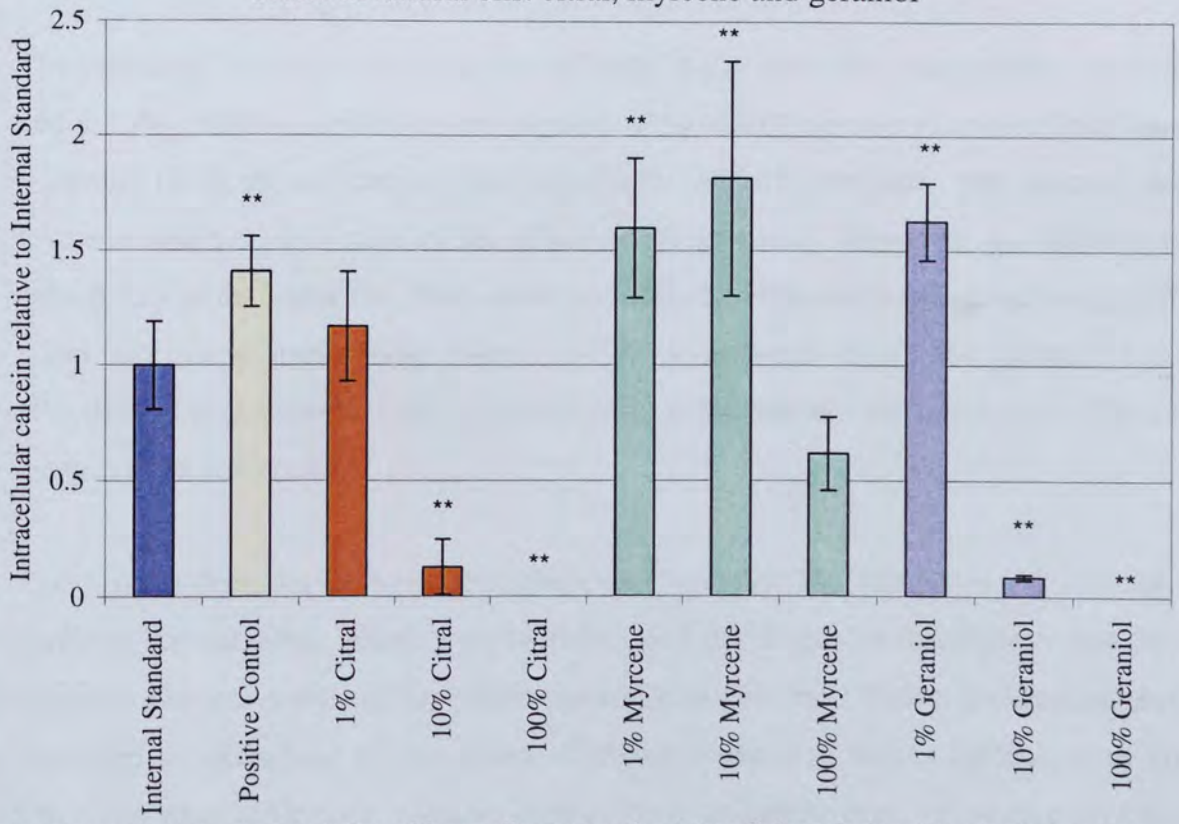
many implications of the lack of effect. The hydroxycinnamic acids can be ruled out as active ingredients in grapefruit juice and other juices and oils. The known clinical benefits of consuming products rich in these acids (*e.g.* antihyperglycemic and antimutagenic properties) must be from mechanisms other than inhibition of p-glycoprotein activity. Antioxidant ability does not necessarily correlate with the ability to inhibit p-glycoprotein.

In summary, the four hydroxycinnamic acids studied have many known properties but had no effect on the activity of p-glycoprotein and it is also unlikely that higher concentrations or closely related compounds would have any effect.

5.3.6 Effect of natural linear compounds

Figure 5.11 below shows the effect on intracellular calcein of the internal standard and various concentrations of three linear compounds found in natural oils: citral, myrcene and geraniol.

Figure 5.11 Effect on intracellular calcein of three linear compounds found in natural oils: citral, myrcene and geraniol



Data are mean and standard deviation of four wells expressed as intracellular calcein relative to the Internal Standard. The data were collected from multiple experiments therefore only one of the positive controls is presented. An ANOVA analysis and Dunnett's post-test were used to identify conditions that had significantly different intracellular calcein levels to the internal standard. ** Denotes a very significant ($p<0.01$) difference from the internal standard.

Both citral and geraniol had similar effects on intracellular calcein at the higher concentrations. A 10% emulsion caused a very significant ($p<0.01$) decrease in intracellular calcein (to approximately 10% of the internal standard result), and a 100% oil was toxic to the cells and caused a very significant ($p<0.01$) decrease in intracellular calcein (to approximately zero).

These results are similar to the effect of grapefruit and bergamot oil seen in Figure 5.7 previously, although the effect of 10% emulsions was greater. In both the previous oils, the 1% emulsions caused a significant increase in intracellular calcein and therefore significant inhibition of p-glycoprotein. Figure 5.13 above shows that geraniol had a similar effect (1% caused a significant ($p<0.01$) increase in intracellular calcein) but citral did not (1% caused no significant ($p>0.05$) effect). It was previously concluded that the other oils did inhibit p-glycoprotein but at higher concentrations they interacted with either the ester or the cells. A lower concentration may inhibit p-glycoprotein without causing any other interaction and might have a significant effect on intracellular calcein. This is potentially true for citral as a 1% emulsion caused a non-significant increase in intracellular calcein.

The effects of myrcene emulsions are different to the other two compounds. Both a 1% and a 10% emulsion caused a very significant ($p<0.01$) increase in intracellular calcein, while the 100% oil only caused a non-significant ($p>0.05$) decrease. The effect of the 1% myrcene emulsion is similar to the effect of 1% geraniol. However, as 100% myrcene caused less of an interaction than 100% geraniol, the 10% emulsion still has a significant effect increasing intracellular calcein. This compound shows the balance between inhibition of p-glycoprotein and interaction with either calcein-AM or the cells differs from compound to compound.

The main differences between the compounds are that 1% emulsions of myrcene and geraniol, but not citral, inhibit p-glycoprotein, and that higher concentrations of citral and geraniol, but not myrcene, abolished intracellular calcein. These differences may be explained by examining the structures of the compounds as shown previously in Figure 5.4. The main difference between then is their oxidation state. Myrcene is a simple hydrocarbon containing ten carbon atoms, geraniol is the related primary alcohol, and citral is the related aldehyde.

The interaction with either the calcein-AM or the cells by citral and geraniol can be explained by the presence in those molecules of an oxygen atom that can be used to make hydrogen bonds. Myrcene cannot form hydrogen bonds so it is less likely to interact. However, the hydrocarbon and the alcohol but not the aldehyde inhibited the activity of p-glycoprotein. This indicates that a degree of hydrophobicity is needed to bind to and inhibit the transporter, and that the position of the oxygen atom in the aldehyde may reduce binding and inhibition.

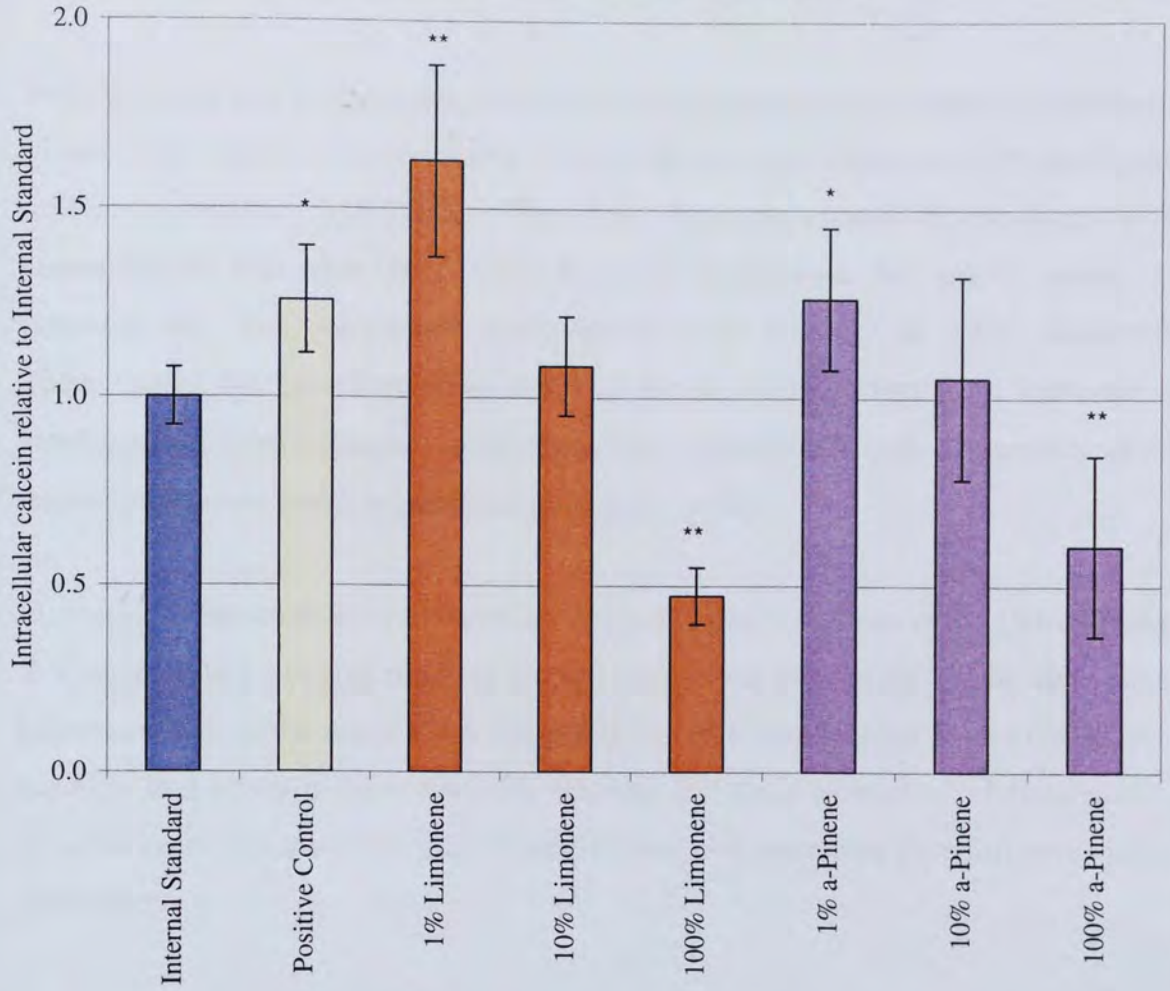
Chronic dosing of citral has a number of effects on the murine liver such as peroxisome proliferation and induction of microsomal CYP4A1 [Roffey *et al.*, 1990]. It can now be concluded that inhibition of p-glycoprotein is unlikely to be one of those effects. All three compounds are found in many plant oils, including grapefruit and bergamot, therefore citral can be ruled out as an active ingredient in the oils while myrcene and/or geraniol may be one of many active compounds in the oil.

In summary, at high concentrations, citral and geraniol interact with either the calcein-AM or the cells while myrcene does not. At lower concentrations, myrcene and geraniol, but not citral, inhibit p-glycoprotein. These results shown that the balance between the ability to inhibit p-glycoprotein and the ability to interact varies from compound to compound. Citral and geraniol interact as the presence of an oxygen atom in their structures allows them to form hydrogen bonds. Citral can be ruled out as an active component of grapefruit juice and oil due to its lack of effect on intracellular calcein at 1%.

5.3.7 Effect of natural cyclic compounds

Figure 5.12 below shows the effect on intracellular calcein of the internal standard and various concentrations of two cyclic compounds found in natural oils: limonene and α-pinene. The effects of emulsions of limonene and α-pinene were very similar. The 1% emulsions significantly ($p<0.05$ or $p<0.01$) increased intracellular calcein. The 10% emulsions had no significant ($p>0.05$) effect, while the 100% oils significantly ($p<0.01$) reduced intracellular calcein (to approximately 50% of the internal standard level). The lack of effect of 10% emulsions is probably due to competing effects on intracellular calcein that cancel each other out or there could simply be no effect.

Figure 5.12 Effect on intracellular calcein of two cyclic compounds found in natural oils: limonene and α-pinene



Data are mean and standard deviation of four wells expressed as intracellular calcein relative to the Internal Standard. The data were collected from multiple experiments therefore only one of the positive controls is presented. An ANOVA analysis and Dunnett's post-test were used to identify conditions that had significantly different intracellular calcein levels to the internal standard. * Denotes a significant ($p<0.05$) difference from the internal standard. ** Denotes a very significant ($p<0.01$) difference from the internal standard.

Examination of the structures of the two compounds (see Figure 5.5 above) shows that they are both hydrocarbons containing ten carbon atoms. They also contain a cyclohexane ring with one double bond. They are similar to myrcene (see Figure 5.4) in that they have ten carbon atoms, although they are different shapes, and are similar to many of the other compounds tested in that they contain a six-member carbon ring although not a phenolic ring like the other compounds (*e.g.* the hydroxycinnamic acids in Figure 5.3).

The addition of 100% oils of both limonene and α-pinene significantly ($p<0.01$) reduced intracellular calcein by approximately 50%. This effect was similar to the effect of 100% myrcene seen in Figure 5.2 but not citral or geraniol. It was previously concluded that the

lack of an oxygen atom in myrcene reduced the molecule's ability to interact with the calcein-AM or the cells. The results presented in Figure 5.12 for 100% limonene and 100% α-pinene support this conclusion.

Both limonene and α-pinene are inhibitors of p-glycoprotein and found in grapefruit juice [Shaw *et al.*, 2001]. Limonene was found at have a concentration of 20 ppm (parts per million) equivalent to 0.002%. Therefore, they are present in the juice at lower concentrations than were tested. This limits the conclusions that can be drawn. It was proposed that both compounds might inhibit more strongly at lower concentrations. Theoretically, the grapefruit effect could be due to a large effect from limonene, and a smaller effect from α-pinene. This seems very unlikely but both compounds should be tested at the levels found in grapefruit juice to be certain.

Limonene is known to have anticarcinogenic properties [Elegbede *et al.*, 1986, Wattenberg & Coccia, 1991] although this was studied using diets containing 5% or 10% limonene. Limonene is found in many fruits and plants and is a widely used flavouring agent. The question that arises is how clinically relevant are these properties of limonene. This question cannot be answered until the activities at concentrations found in citrus juices are determined.

In summary, like a number of the previously tested compounds, limonene and α-pinene inhibited at 1% but interacted with either calcein-AM or the cells at 100%. However, 100% oils only reduced calcein accumulation by 50%, a similar effect to myrcene. This result supported the proposed theory that hydrophobic compounds without an oxygen atom interacted less than compounds containing oxygen. Limonene was a significantly more potent inhibitor of the activity of p-glycoprotein than α-pinene, indicating the importance of the three-dimensional arrangement of atoms for inhibition. Both compounds are constituents of grapefruit juice and both therefore contribute to the activity of the juice. However, the proportion of the activity due to each compound will remain unknown until both compounds are tested at the relevant concentrations.

5.4 Conclusion

The aim of this chapter was to investigate the effect of a range of natural compounds on the activity of p-glycoprotein. The first objective was to identify the effect of grapefruit juice on the modified calcein assay. The juice needed to be buffered to pH 7 before any inhibition was identified, however the intestinal buffering of the contents of the stomach as it passes through the small intestine means that this result could be clinically relevant. The activity of other juices (lemon and orange) and oils (grapefruit and bergamot) was then examined to reduce the number of candidate active constituents in grapefruit juice.

Buffering the fruit juices had no significant effect on p-glycoprotein but emulsions (1%) of both the oils tested significantly inhibited p-glycoprotein. Therefore, it can be concluded that compounds that are components of grapefruit juice and either orange or lemon juice were very unlikely to be the active ingredients in grapefruit juice while compounds that are components of grapefruit juice and either grapefruit or bergamot oil are potentially the active ingredients in grapefruit juice.

Both grapefruit and bergamot oil abolished intracellular calcein when tested at 100%. The calcein-AM may partition more in the hydrophobic oil than the PBS therefore reducing the amount available to enter the cells and reducing the possible intracellular calcein. Another possibility is that the hydrophobic oil may also have interacted with the cell membranes and disrupted them.

Previous studies have found a correlation between substrates for CYP3A4 and substrates for p-glycoprotein. The flavone derivatives are known to be substrates for CYP3A4 and some are found in citrus juices. However, naringenin, naringin and hesperidin had no significant effect on intracellular calcein. Naringin and naringenin are found in grapefruit juice but at concentrations found to have no effect on the activity of p-glycoprotein. Hesperidin is a major component of orange juice (65 mg/L). Its inactivity correlated with the inactivity of orange juice. Three non-citrus flavone derivatives were also tested. Genistein inhibited p-glycoprotein at a high concentration, a result consistent with other studies. Kaempferol and rutin are known to have a range of properties but have no effect on p-glycoprotein.

Other known inhibitors of CYP3A4 are the furanocoumarins. Four furanocoumarins (5-methoxypsoralen, 8-methoxypsoralen, trioxsalen and khellin) were found to be poorly

soluble and therefore unable to be tested. 5-methoxypsoralen (5-MOP) is found in grapefruit juice at a concentration of 1 mg/L (~4 μM) [Ho *et al.*, 2000] yet it is such a potent inhibitor of CYP3A4 that it is thought to be the major contributor of the effect of grapefruit juice on CYP3A4 [Ho *et al.*, 1998]. The furanocoumarins remain the components of grapefruit juice that are most likely to inhibit the activity of p-glycoprotein yet their poor solubility remains a barrier to proving this.

Four hydroxycinnamic acids were studied as they have antioxidant properties similar to some known inhibitors of p-glycoprotein. They had no effect on the activity of p-glycoprotein and it is also unlikely that higher concentrations or closely related compounds would have any effect.

Most inhibitors of p-glycoprotein are hydrophobic. Three natural linear compounds (citral, geraniol and myrcene) were studied to identify the effect of their oxidation state on the potency of inhibition. Citral had no inhibitory activity, while myrcene was more potent than geraniol. The results showed that the balance between the ability to inhibit p-glycoprotein and the ability to interact with calcein-AM or the cells varied from compound to compound. The presence of an oxygen atom allows the formation of hydrogen bonds to increase the interaction, however it may also reduce the molecule's ability to bind to and inhibit p-glycoprotein. Citral was also ruled out as an active component of grapefruit juice.

Two cyclic natural compounds were studied to identify the effect of the three-dimensional arrangements of the molecule on its ability to inhibit p-glycoprotein. Like a number of the previously studied compounds, limonene and α-pinene inhibited at 1% but were toxic at 100%. The results supported the proposed theory that hydrophobic compounds without an oxygen atom interact with calcein-AM or the cells less than compounds containing oxygen. Limonene was significantly more potent than α-pinene indicating the importance of the three-dimensional arrangement of atoms for inhibition. Both compounds would contribute to the activity of grapefruit juice. However, the proportion of the activity due to each compound will remain unknown until both compounds are tested at clinically relevant concentrations.

The modified assay has clearly shown that a range of unrelated compounds, juices and oils have a range of potencies for the inhibition of p-glycoprotein. The effect of (buffered) grapefruit juice on the assay correlates with previous studies *in vivo* and *in vitro* (see

Chapter 1: Introduction for summary). The juice was by far the strongest inhibitor tested. The juice could be further studied at a range of concentrations to identify any dose-related effect. Rehydrated grapefruit puree had no effect but more studies need to be performed to find out which hypothesis for this result is true. The lack of effect of the other citrus juices also correlated with clinical studies. The effects of fruit oils were studied to help identify likely active constituents of grapefruit juice. A range of other naturally occurring compounds (flavone derivatives, hydroxycinnamic acids, and natural linear and cyclic compounds) were tested to reduce the number of potential active constituents of grapefruit juice.

Two sets of experiments need to be performed in the future to confirm the conclusions from the juices and oils sections (5.3.1 and 5.3.2). First of all, other solutions with a pH of 3 can be buffered to pH 7 to determine that the effects of grapefruit juice at those pH values was due to the components of the juice and not simply the pH. The second set of experiments involve the testing of other oils (such as olive or corn oil) to determine that increase in intracellular calcein at 1% was due to the components of the oils and not due to their hydrophobicity. It would be expected that these other oils would interact with the calcein-AM or cells at 100% in a similar manner to the hydrophobic oils and compounds tested in this study.

To identify the active constituents of grapefruit juice, the juice could be fractionated and each fraction tested for activity. Each fraction can then be examines to identify all of its constituents. A long-term aim would be to have a list of every constituent of the juice, their concentrations and their contribution to the inhibition of p-glycoprotein at that concentration.

All the oils studied should be tested at lower concentrations. There seems to be a balance between activity and toxicity. The results presented here generally have activity at only one concentration, No trends can be identified with only one data point. Therefore each oil should be studied at a range of lower concentrations to clarify the concentration-activity relationship. A control oil (*e.g.* paraffin oil) could be used to identify effects due to oils rather than other constituents.

The assay could be used to identify the effects of compounds and excipients on intracellular calcein to develop formulations that would inhibit the activity of p-

glycoprotein. The assay would effectively be a screen for inhibitory activity and formulations could then undergo more detailed studies to quantify their effects.

6. CONCLUSION

ABSTRACT:

The investigations into the structure-activity relationships for the intestinal dipeptide transporter, the development and validation of the calcein-AM assay and the inhibitory activity of natural compounds are discussed. The implications of these studies for designing new therapies, specifically against HIV, are also examined.

The aim of this thesis was to study mechanistic aspects of absorption and efflux to inform drug design and *in vitro* testing. The first part investigated increasing drug uptake across the cell membrane into the cell. The second part investigated drug efflux back into the intestine and identify ways of reducing it.

The investigation of the dipeptide transporter found that the optimum conditions for measuring the inhibition of Gly-Pro uptake were after three minutes, at pH 6, at 37°C and with DMSO at less than 2%. Proline was also added although it had no significant effect on uptake. Other experiments showed that uptake was concentration–dependent and inhibited by both dipeptides and tripeptides, as expected.

Structure-activity relationships for the intestinal dipeptide transporter were investigated by developing a rapid screening system and using it to determine the affinities of a number of dipeptide and non-dipeptide derivatives. The derivatives were then grouped into series leading to the identification of a number of structure-activity relationships. A statistical analysis correlating IC_{50} with molecular parameters only found significant relationships with the heat of formation and number of hydrogen-bond acceptor atoms in the molecule. A second analysis found significant links between IC_{50} and the size of the molecule, R_2 and R_3 groups and the dipole moment of the molecule. These results indicated that while molecular properties did affect IC_{50}, the size, charge and composition of the R_1, R_2 and R_3 groups caused the most significant effects. These results can be used to inform prodrug design.

The second part of the thesis investigated p-glycoprotein. An *in vitro* assay for the transporter was developed. Two modifications to the calcein accumulation assay were proposed: quenching with cobalt chloride and preincubating the test solutions with the cells. Cobalt chloride has clearly been shown to quench the fluorescence of calcein. It could be used to quench extracellular calcein to allow the measurement of intracellular calcein, removing the need for a plate centrifuge. A preincubation stage increased the significance of the difference between the internal standard and positive control. The effect on intracellular calcein was identical for the internal standard and negative control (mannitol) however the positive control (cyclosporin A) significantly increased intracellular calcein.

The assay was then validated by determining the effect of known inhibitors of p-glycoprotein, compounds that are known to have no significant effects on the transporter and inhibitors of MRP. Two known inhibitors of p-glycoprotein were tested and their potencies compared to cyclosporin A, used as a positive control. They all significantly increased intracellular calcein. The order of potencies were cyclosporin A > terfenadine > verapamil. This order correlates well with previously published studies. It was concluded that the assay detected inhibitors of p-glycoprotein and allowed their potencies to be ranked. The effects of verapamil, buthionine sulfoximine and nifedipine on the transport of vincristine were studied to check the specificity of the assay. The studies agreed with the assay results for verapamil and buthionine sulfoximine but differed with regard to the effect of nifedipine. This difference is however reflected in the literature. Taken together, the experiments in this chapter prove that the modified calcein accumulation assay specifically identifies inhibitors of p-glycoprotein as MRP activity is present but not significant. The assay can now be used to identify any inhibitory effects of compounds on p-glycoprotein activity. If the protocol is altered in the future then the panel of experiments performed here can be repeated to confirm the specificity of the assay.

The modified protocol now allows the identification of the effect of compounds (in solution) on the activity of p-glycoprotein using a simple cell suspension instead of growing cell monolayers. The assay can be used as a screen for inhibitory activity with other more time-consuming techniques then used to quantify the relative size of the effect. There are a number of potential future modifications to improve the efficiency and usefulness of the assay. These include using cells that have been modified to express high levels of p-glycoprotein to increase the difference between the positive control and the internal standard and automating the system to reduce variations due to human error.

The final objective was to identify the effect of grapefruit juice and other natural compounds on p-glycoprotein. Grapefruit juice needed to be buffered to pH 7 before any inhibition was identified, however the intestinal buffering of the contents of the stomach as it passes through the small intestine means that this result could be clinically relevant. Buffering orange and lemon juices had no significant effect on p-glycoprotein but emulsions (1%) of both grapefruit and bergamot oils tested significantly inhibited p-glycoprotein. Therefore, it was concluded that compounds that are components of grapefruit juice and either orange or lemon juice were very unlikely to be the active ingredients in grapefruit juice while compounds that are components of grapefruit juice

and either grapefruit or bergamot oil are potentially the active ingredients in grapefruit juice. Both grapefruit and bergamot oil abolished intracellular calcein when tested at 100%. The calcein-AM may partition more in the hydrophobic oil than the PBS therefore reducing the amount available to enter the cells and reducing the possible intracellular calcein. Another possibility is that the hydrophobic oil may also have interacted with the cell membranes and disrupted them.

Previous studies have found a correlation between substrates for CYP3A4 and substrates for p-glycoprotein. The flavone derivatives are known to be substrates for CYP3A4 and some are found in citrus juices. However, naringenin, naringin and hesperidin had no significant effect on intracellular calcein. Three non-citrus flavone derivatives were also tested. Genistein inhibited p-glycoprotein at a high concentration, a result consistent with other studies. Kaempferol and rutin are known to have a range of properties but were found to have no effect on p-glycoprotein. Other known inhibitors of CYP3A4 are the furanocoumarins. Four furanocoumarins (5-methoxypsoralen, 8-methoxypsoralen, trioxsalen and khellin) were found to be poorly soluble and therefore unable to be tested. The furanocoumarins remain the components of grapefruit juice that are most likely to inhibit the activity of p-glycoprotein yet their poor solubility remains a barrier to proving this. Four hydroxycinnamic acids were studied as they have antioxidant properties similar to some known inhibitors of p-glycoprotein. They had no effect on the activity of p-glycoprotein and it is also unlikely that higher concentrations or closely related compounds would have any effect.

Most inhibitors of p-glycoprotein are hydrophobic. Three natural linear compounds (citral, geraniol and myrcene) were studied to identify the effect of their oxidation state on the potency of inhibition. Citral had no inhibitory activity, while myrcene was more potent than geraniol. The results showed that the balance between the ability to inhibit p-glycoprotein and the ability to interact with calcein-AM or the cells varied from compound to compound. Two cyclic natural compounds were studied to identify the effect of the three-dimensional arrangements of the molecule on its ability to inhibit p-glycoprotein. Like a number of the previously studied compounds, limonene and α-pinene inhibited at 1% but were toxic at 100%. The results supported the proposed theory that hydrophobic compounds without an oxygen atom interact with calcein-AM or the cells less than compounds containing oxygen. Limonene was significantly more potent than α-pinene indicating the importance of the three-dimensional arrangement of atoms for inhibition.

Both compounds would contribute to the activity of grapefruit juice. However, the proportion of the activity due to each compound will remain unknown until both compounds are tested at clinically relevant concentrations.

The experiments have led to the identification of an assay for the screening of compounds that may inhibit p-glycoprotein and also the identification of safe natural compounds that could be co-consumed with a drug to increase the absorption of the drug.

The implications of naturally occurring compounds with the ability to inhibit p-glycoprotein are very significant. The activity of p-glycoprotein (in conjunction with CYP3A4) is a significant barrier to the absorption of a large number of drugs, for example the HIV protease inhibitor saquinavir.

A study of saquinavir pharmacokinetics in seven HIV-infected men [Merry *et al.*, 1997] found a twelve-fold variability in the area under the saquinavir concentration-time curve (0-8 hours). A dose of 600 mg three times a day resulted in AUCs ranging from 293-3446 ng.h/ml and a median C_{max} of 146 ng/ml. However, the co-administration of ritonavir (300 mg twice a day) resulted in AUCs from 7357-108001 ng.h/ml and a median C_{max} of 4795 ng/ml. Both the AUC and C_{max} were significantly higher in the presence of ritonavir. It was noted that the saquinavir dosing schedule for some patients resulted in low plasma levels and possibly little antiviral effect. Combination therapy resulted in a significant drug interaction exposing patients to very high concentrations of saquinavir and potential toxicity. Both drugs are substrates for CYP3A4 and p-glycoprotein and this study showed the significant difference inhibiting these proteins had on saquinavir pharmacokinetics. The aim of the study was to improve the absorption of saquinavir, this aim was achieved but with the side effects being possibly toxic levels of saquinavir and also the likely presence of ritonavir in the blood, however the hypothesis of inhibiting CYP3A4 and p-glycoprotein to increase absorption was proved. This opened up the idea of the co-administration of other CYP3A4/p-glycoprotein inhibitors with saquinavir. More studies were performed on the effect of ritonavir co-administered with other protease inhibitors however the field was fraught with commercial implications as each company struggled to produce data that support higher doses of its own drug and less ritonavir.

The viral load test has been developed to measure the amount of HIV RNA in the blood [Christopherson *et al.*, 1998]. This test has allowed the monitoring of the responses of

patients to antiviral therapies. Studies have shown that three-drug combinations of anti-HIV drugs are much more effective than one or two drug combinations in preventing disease progression and deaths due to AIDS. In combination therapy, patients receive a protease inhibitor (*e.g.* saquinavir or ritonavir) combined with two or more nucleoside reverse transcriptase inhibitors (NRTIs) (*e.g.* AZT or ddI), or another protease inhibitor (*e.g.* nelfinavir), or another protease inhibitor plus two NRTIs or two non-nucleoside reverse transcriptase inhibitors (NNRTIs) (*e.g.* nevirapine or delavirdine) [Figgitt & Plosker, 2000].

The efficiency of combination therapy has been proposed to be due to the different mechanisms of action of the protease inhibitors, NRTIs and NNRTIs. However increased drug absorption though the inhibition of CYP3A4 and p-glycoprotein probably plays a major role. Unfortunately, the emergence of drug-resistant variants of HIV-1 will severely limit the long-term effectiveness of these drugs. HIV can become resistant to the effects of anti-HIV drugs that patients have previously taken. Ideally combination therapy should contain three drugs that the patient has not previously taken. However there are only so many drugs available and HIV is then exposed to three drugs at a time.

Current research is examining every molecular process used by HIV to enter cells and reproduce with the aim of developing new drugs that target HIV at different places to protease and reverse transcriptase inhibitors. Another important point to note it that all these therapies and drugs cost large amounts of money while most HIV+ patients live in third world countries with no money to buy food let alone expensive drugs. Combination therapy has led to the need to take different tablets at different times throughout the day. Few patients stick rigidly to their proscribed dosing schedule, as they are very complicated.

What potential use is the work presented in this thesis? The assay has identified a number of naturally occurring compounds that are inhibitors of p-glycoprotein (including genistein, myrcene, geraniol, limonene and α-pinene). It can also be used to identify many other compounds. These compounds could be synthesised and formulated together with any drug that is affected by p-glycoprotein. The compounds would temporarily inhibit p-glycoprotein so the absorption of the drug would increase significantly. This would reduce the amount of drug needed to be delivered to produce a certain blood concentration. The cost of the tablet would be reduced due to the lower amount of drug used. The naturally occurring compounds are generally quite simple molecules and so they could be

synthesised relatively easily and cheaply. They have been consumed for thousands of years and so there would be fewer regulatory processes to undergo to receive licence approval. All these factors should reduce cost of producing the tablets and hopefully the price they are sold at. The compounds with the largest effect on intracellular calcein were limonene and myrcene.

Studies have shown that the grapefruit juice can be consumed before a drug was consumed (*i.e.* not co-formulated) [Lundahl *et al.*,1995] and still affect the drug pharmacokinetics. However, patients are not very good at sticking to their dosing schedule and so a combined tablet would improve patient compliance. Another potential problem would be simply just due to inhibiting p-glycoprotein. This potentially would allow more drugs and undesirable substances into the body and into cells. It would also potentially circumvent blood-brain and maternal-foetal barriers that provide special protection in these vital areas. The expression of p-glycoprotein reduces the infectivity of HIV throughout the HIV life cycle from the fusion of viral and plasma membranes onwards [Lee *et al.*, 2000]. The benefits of inhibiting p-glycoprotein for drug absorption need to be weighed against the reduced protection to HIV.

The conclusions of the work on the dipeptide transporter also have implications in this area. The identification of the dipeptide and non-dipeptide derivatives that inhibited Gly-Pro uptake have indicated the promiscuity of this transporter. Further work is needed to see if the derivatives are also substrates. If they are then the field of peptide prodrugs can be expanded. Valacyclovir is a peptide prodrug created by the addition of a valyl group to acyclovir. The structure-activity relationships identified in Chapter 3 could be used to inform the modification of the valyl group to a structure that is absorbed more easily.

In summary, the two approaches to increasing drug absorption (chemical modification for recognition by the dipeptide transporter and inhibition of the activity of p-glycoprotein) have major implications for future formulation of medicines. There is a huge potential for the use of natural compounds to be inhibitors of p-glycoprotein. Previous research into anti-HIV drugs has shown the potential of using peptide prodrugs and using drug interactions to boost the efficiency of therapies but also the potential problems that will be faced.

REFERENCES

Abdel-Rahman, S. M., Lan L., Yasuda K., Leeder J. S., Kearns G. L., and Schuetz E. G. (2000). Cisapride (CIS) is an inhibitor but not a substrate for human P-glycoprotein. *Clinical Pharmacology & Therapeutics* 67(2): PI-34.

Abedel-nasser, A. G. , Simons C., and Gumbleton M. (2000). Increased intracellular concentration of P-glycoprotein substrates in multidrug resistant cells by molecules isolated from grapefruit oil. *British Journal Of Pharmacology* 131: 126P.

Addison, J. M., Burston D., Dalrymple J. A., Mattwas D. M., Payne J. W., Sleisinger M. H., and Wilkinson S. (1975). A common mechanism for transport of di- and tri-peptides by hamster jejunum in vitro. *Clinical Science and Molecular Medicine* 49: 313-322.

Adibi, S. A. (1997). The oligopeptide transporter (Pept-1) in human intestine: Biology and function. *Gastroenterology* 113: 332-340.

Akiyama, T. , Ishida J., Nakagawa S., Ogawara H., Watanabe S., Itoh N., Shibuya M., and Fukami Y. (1987). Genistein, a Specific Inhibitor Of Tyrosine-Specific Protein-Kinases. *Journal Of Biological Chemistry* 262(12): 5592-5595.

Ambudkar, S.V., Dey S., Hrycyna C.A., Ramachandra M., Pastan I., and Gottesman M. M. (1999). Biochemical, cellular, and pharmacological aspects of the multidrug transporter. *Annual Reviews in Pharmacology and Toxicology* 39: 361-398.

Ameer, B. , Weintraub R. A., Johnson J. V., Yost R. A., and Rouseff R. L. (1996). Flavanone absorption after naringin, hesperidin, and citrus administration. *Clinical Pharmacology & Therapeutics* 60(1): 34-40.

Amidon, G. L. & Lee H. J. (1994). Absorption of peptide and peptidomimetic drugs. *Annual Reviews in Pharmacology and Toxicology* 34: 321-341.

Aoyama, T. , Gonzalez F. J., and Gelboin H. V. (1989). Mutagen Activation By Cdna-Expressed P1450, P3450, and P450a. *Molecular Carcinogenesis* 1(4): 253-259.

Ariyoshi, T., Arakaki M., Ideguchi K., Ishizuka Y., and Noda K. (1975). Studies on the metabolism of d-Limonene (p-Mentha-1,8-diene). III. Effects of d-Limonene on the lipids and drug-metabolizing enzymes in rat livers. *Xenobiotica* 5(1): 33-38.

Artursson, P. (1989). Epithelial transport of drugs in cell culture. I: A model for studying the passive diffusion of drugs over intestinal absorbtive (Caco-2) cells. *Journal of Pharmaceutical Sciences* 79(6): 476-482.

Artursson, P. & J. Karlsson (1991). Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. *Biochemical and Biophysical Research Communications* 175(3): 880-885.

Artursson, P. & R. T. Borchardt (1997). Intestinal drug absorption and metabolism in cell cultures: Caco-2 and beyond. *Pharmaceutical Research* 14(12): 1655-1658.

Azuma, J. , Yamamoto I., Watase T., Orii Y., Tanigawa T., Terashima S., Yoshikawa K., Tanaka T., and Kawano K. (1998). Effects of grapefruit juice on the pharmacokinetics of the calcium channel blockers nifedipine and nisoldipine. *Current Therapeutic Research - Clinical and Experimental* 59(9): 619-634.

Bai, P., Subramanian P., Mosberg H. I., and Amidon G. L. (1991). Structural requirements for the intestinal mucosal-cell peptide transporter: The need for N-terminal a-amino group. *Pharmaceutical Research* 8(5): 593-599.

Bai, J. P. F., Hu M., Subramanian P., Mosberg H. I., and Amidon G. L. (1992). Utilization of peptide carrier system to improve intestinal absorption: Targeting prolidase as a prodrug-converting enzyme. *Journal of Pharmaceutical Sciences* 81(2): 113-116.

Bailey, D. G., Edgar B., Spence J. D., Munoz C., and Arnold J. M. O. (1990). Felodipine and Nifedipine Interactions With Grapefruit Juice. *Clinical Pharmacology & Therapeutics* 47(2): 180.

Bailey, D. G. , Spence J. D., Munoz C., and Arnold J. M. O. (1991). Interaction of citrus juices with felodipine and nifedipine. *Lancet* 337: 268-269.

Bailey, D. G., Munoz C., Arnold J. M. O., Strong H. A., and Spence J. D. (1992). Grapefruit Juice and Naringin Interaction With Nitrendipine. *Clinical Pharmacology & Therapeutics* 51(2): 156.

Bailey, D. G., Arnold J. M. O., Munoz C., and Spence J. D. (1993). Grapefruit Juice Felodipine Interaction - Mechanism, Predictability, and Effect Of Naringin. *Clinical Pharmacology & Therapeutics* 53(6): 637-642.

Bailey, D. G., Arnold J. M. O., Tran L. T., Ahktar J., and Spence J. D. (1994). Marked Effects Of Both Erythromycin and Grapefruit Juice On Felodipine Pharmacokinetics. *Clinical Pharmacology & Therapeutics* 55(2): 165.

Bailey, D. G., Arnold J. M. O., Bend J. R., Tran L. T., and Spence J. D. (1995). Grapefruit Juice-Felodipine Interaction - Reproducibility and Characterization With the Extended-Release Drug Formulation. *British Journal Of Clinical Pharmacology* 40(2): 135-140.

Bailey, D. G. , Bend J. R., Arnold J. M. O., Tran L. T., and Spence J. D. (1996). Erythromycin-felodipine interaction: Magnitude, mechanism, and comparison with grapefruit juice. *Clinical Pharmacology & Therapeutics* 60(1): 25-33.

Bailey, D. G. , Munoz C., Kreeft J. H., Freeman D. J., and Bend J. R. (1998a). Grapefruit juice felodipine interaction: Amount and effect of active ingredients in juice fractions in man. *Clinical Pharmacology & Therapeutics* 63(2): PI99.

Bailey, D. G. , Kreeft J. H., Munoz C., Freeman D. J., and Bend J. R. (1998b). Grapefruit juice felodipine interaction: Effect of naringin and 6 ',7 '-dihydroxybergamottin in humans. *Clinical Pharmacology & Therapeutics* 64(3): 248-256.

Bailey, D. G. , Dresser G. R., Kreeft J. H., Munoz C., Freeman D. J., and Bend J. R. (2000). Grapefruit-felodipine interaction: Effect of unprocessed fruit and probable active ingredients. *Clinical Pharmacology & Therapeutics* 68(5): 468-477.

Baliman, P. V., Tamai I., Guo A., Nakanishi T., Kitada H., Leibach F. H., Tsuji A., and Sinko P. J. (1998). Direct evidence for peptide transporter (PepT1)-mediated uptake of a nonpeptide prodrug, valacyclovir. *Biochemical and Biophysical Research*

Communications 250: 246-251.

Baltes, M. R. H. , Dubois J. G., and Hanocq M. (2001). Application to drug-food interactions of living cells as in vitro model expressing cytochrome P450 activity: enzyme inhibition by lemon juice. *Talanta* 54(5): 983-987.

Barfuss, D. W., Ganapathy V., and Leibach F. H. (1988). Evidence For Active Dipeptide Transport In Isolated Proximal Straight Tubules. *American Journal Of Physiology* 255(1): F177-F181.

Becquemont, L. , Verstuyft C., Kerb R., Brinkmann U., Lebot M., Jaillon P., and Funck-Brentano C. (2001). Effect of grapefruit juice on digoxin pharmacokinetics in humans. *Clinical Pharmacology & Therapeutics* 70(4): 311-316.

Benet, L. Z., Wu C., Hebert M. F., and Wacher V. J. (1996). Intestinal drug metabolism and antitransport processes: A potential paradigm shift in oral drug delivery. *Journal of Controlled Release* 39: 139-143.

Benton, R. E. , Honig P. K., Zamani K., Cantilena L. R., and Woosley R. L. (1996). Grapefruit juice alters terfenadine pharmacokinetics resulting in prolongation of repolarization on the electrocardiogram. *Clinical Pharmacology & Therapeutics* 59(4): 383-388.

Bethea, D. , Fullmer B., Syed S., Seltzer G., Tiano J., Rischko C., Gillespie L., Brown D., and Gasparro F. P. (1999). Psoralen photobiology and photochemotherapy: 50 years of science and medicine. *Journal Of Dermatological Science* 19(2): 78-88.

Boll, M. , Herget M., Wagener M., Weber W. M., Markovich D., Biber J., Clauss W., Murer H., and Daniel H. (1996). Expression cloning and functional characterization of the kidney cortex high-affinity proton-coupled peptide transporter. *Proceedings Of the National Academy Of Sciences Of the United States Of America* 93(1): 284-289.

Borst, P. , Evers R., Kool M., and Wijnholds J. (1999). The multidrug resistance protein family. *Biochimica Et Biophysica Acta-Biomembranes* 1461(2): 347-357.

Brogden, R. N. & Benfield P. (1996). Verapamil - A review of its pharmacological properties and therapeutic use in coronary artery disease. *Drugs* 51(5): 792.

Buchler, M. , Konig J., Brom R., Kartenbeck J., Spring H., Horie T., and Keppler D. (1996). cDna cloning of the hepatocyte canalicular isoform of the multidrug resistance protein, cMrp, reveals a novel conjugate export pump deficient in hyperbilirubinemic mutant rats. *Journal Of Biological Chemistry* 271(25): 15091-15098.

Burggraaf, J. , Schoemaker R., and Cohen A. F. (1997). The effect of grapefruit juice (GJ) on the pharmacokinetics (PK) and pharmacodynamics (PD) of nifedipine administered in two different formulations. *Clinical Pharmacology & Therapeutics* 61(2): IV66.

Burgos, F. & R.C. Capone (1996). Ocular and systemic manifestations of gout. *Clinical Eye and Vision Care* 8: 155-163.

Calhau, C. , Martel F., Hipolito-Reis C., and Azevedo I. (2000). Effect of P-glycoprotein modulators on alkaline phosphatase activity in cultured rat hepatocytes. *Cellular Physiology and Biochemistry* 10(4): 195-202.

Castro, A. F. & Altenberg G. A. (1997). Inhibition of drug transport by genistein in multidrug- resistant cells expressing P-glycoprotein. *Biochemical Pharmacology* 53(1): 89-93.

Castro, N. , Jung H., Medina R., Gonzalez-Esquivel D., Lopez M., and Sotelo J. (2002). Interaction between grapefruit juice and praziquantel in humans. *Antimicrobial Agents and Chemotherapy* 46(5): 1614-1616.

Cavet, M. E. , West M., and Simmons N. L. (1996). Transport and epithelial secretion of the cardiac glycoside, digoxin, by human intestinal epithelial (Caco-2) cells. *British Journal Of Pharmacology* 118(6): 1389-1396.

Chen, X. Z. , Zhu T., Smith D. E., and Hediger M. A. (1999). Stoichiometry and kinetics of the high-affinity H+-coupled peptide transporter PepT2. *Journal Of Biological Chemistry* 274(5): 2773-2779.

Cheng, K. L. Nafziger A. N. Peloquin C. A. Amsden G. W. (1998). Effect of grapefruit juice on clarithromycin pharmacokinetics. *Antimicrobial Agents and Chemotherapy* 42(4): 927.

Cheng, J. T. & Liu I. M. (2000). Stimulatory effect of caffeic acid on alpha(1A)-adrenoceptors to increase glucose uptake into cultured C2C12 cells. *Naunyn-Schmiedebergs Archives Of Pharmacology* 362(2): 122-127.

Christopherson, C., Lu S. D., and Kwok S. (1998). Laboratory markers of antiviral activity. *Antiviral Therapeutics* 3(4): 247-250.

Chuan, M. T. , Tsai Y. J., and Wu M. C. (1999). Effectiveness of psoralen photochemotherapy for vitiligo. *Journal Of the Formosan Medical Association* 98(5): 335-340.

Clifford, C. P. , Adams D. A., Murray S., Taylor G. W., Wilkins M. R., Boobis A. R., and Davies D. S. (1997). The cardiac effects of terfenadine after inhibition of its metabolism by grapefruit juice. *European Journal of Clinical Pharmacology* 52: 311-315.

Clifford, M. N. (1999). Chlorogenic acids and other cinnamates - nature, occurrence and dietary burden. *Journal Of the Science Of Food and Agriculture* 79(3): 362-372.

Cole, S. P. C., Bhardwaj G., Gerlach J. H., Mackie J. E., Grant C. E., Almquist K. C., Stewart A. J., Kurz E. U., Duncan A. M. V., and Deeley R. G. (1992). Overexpression Of a Transporter Gene In a Multidrug-Resistant Human Lung-Cancer Cell-Line. *Science* 258(5088): 1650-1654.

Conseil, G. , Baubichon-Cortay H., Dayan G., Jault J. M., Barron D., and Di Pietro A. (1998). Flavonoids: A class of modulators with bifunctional interactions at vicinal ATP- and steroid-binding sites on mouse P-glycoprotein. *Proceedings Of the National Academy Of Sciences Of the United States Of America* 95(17): 9831-9836.

Critchfield, J.W., Welsh C.J., Phang J.M., and Yeh G.C. (1994). Modulation Of Adriamycin(R) Accumulation and Efflux By Flavonoids In Hct-15 Colon Cells -

Activation Of P-Glycoprotein As a Putative Mechanism. *Biochemical Pharmacology* 48(7): 1437-1445.

Croud, V. B., Michaelis J. R., and Pindar A. G. (1983). Isolation Of Bergapten and Limettin From Bergamot Oil. *Analyst* 108(1293): 1532-1534.

Cundy, K. C. , Li Z. H., and Lee W. A. (1996). Effect of probenecid on the distribution, metabolism, and excretion of cidofovir in rabbits. *Drug Metabolism and Disposition* 24(3): 315-321.

Cvetkovic, M. , Leake B., Fromm M. F., Wilkinson G. R., and Kim R. B. (1999). OATP and P-glycoprotein transporters mediate the cellular uptake and excretion of fexofenadine. *Drug Metabolism and Disposition* 27(8): 866-871.

Dalton, W.S., Grogan T.M., Rybski J.A., Scheper R.J., Richter L., Kailey J., Broxterman H.J., Pinedo H.M., and Salmon S.E. (1989). Immunohistochemical Detection and Quantitation Of P- Glycoprotein In Multiple Drug-Resistant Human Myeloma Cells - Association With Level Of Drug-Resistance and Drug Accumulation. *Blood* 73(3): 747752.

Damkier, P. , Hansen L. L., and Brosen K. (1999). Effect of diclofenac, disulfiram, itraconazole, grapefruit juice and erythromycin on the pharmacokinetics of quinidine. *British Journal Of Clinical Pharmacology* 48(6): 829-838.

Daniel, H, Morse E. L., and Adibi S. A. (1992). Determinants of substrate affinity for the oligopeptide/H^+ symporter in the renal brush border membrane. *The Journal of Biological Chemistry* 267(14): 9565-9573.

Dantzig, A. H. &'Bergin L. (1990). Uptake of cephalosporin, cephalexin, by a dipeptide transport carrier in the human intestinal cell line, Caco-2. *Biochimica et Biophysica Acta* 1027(211-217).

Dantzig, A. H., Hoskins J., Tabas L. B., Bright S., Shepard R. L., Jenkins I. L., Duckworth D. C., Sportsman J. R., Mackensen D., Rosteck P. R., and Skatrud P. L. (1994). Association of intestinal peptide transport with a protein related to the cadherin

superfamily. *Science* 264: 430-433.

Davies, A.J., Harindra V., McEwan A., and Ghose R.R. (1989). Cardiotoxic Effect With Convulsions In Terfenadine Overdose. *British Medical Journal* 298(6669): 325.

de Vries, M. H., Hofman G. A., Koster A. S. J., and Noordhoek J. (1989). Systemic Intestinal Metabolism Of 1-Naphthol - a Study In the Isolated Vascularly Perfused Rat Small-Intestine. *Drug Metabolism and Disposition* 17(5): 573-578.

de Vrueh, R. L. A. , Smith P. L., and Lee C. (1998). Transport of L-valine-acyclovir vio the oligopeptide transporter in the human intestinal cell line, caco-2. *Journal of Pharmacology and Experimental Therapeutics* 286(3): 1166-1170.

de Waziers, I. , Cugnenc P. H., Yang C. S., Leroux J. P., and Beaune P. H. (1990). Cytochrome-P-450 Isoenzymes, Epoxide Hydrolase and Glutathione Transferases In Rat and Human Hepatic and Extrahepatic Tissues. *Journal Of Pharmacology and Experimental Therapeutics* 253(1): 387-394.

Delie, F. & Rubas W. (1997). A human colonic cell line sharing similarities with enterocytes as a model to examine oral absorption: Advantages and limitations of the Caco-2 model. *Critical Reviews in Therapeutic Drug Carrier Systems* 14(3): 221-286.

Dresser, G. K. , Bailey D. G., and Carruthers S. G. (2000). Grapefruit juice-felodipine interaction in the elderly. *Clinical Pharmacology & Therapeutics* 68(1): 28-34.

Dresser, G. K. , Bailey D. G., Leake B. F., Schwarz U. I., Dawson P. A., Freeman D. J., and Kim R. B. (2002). Fruit juices inhibit organic anion transporting polypeptide-mediated drug uptake to decrease the oral availability of fexofenadine. *Clinical Pharmacology, & Therapeutics* 71(1): 11-20.

Ducharme, M. P., Warbasse L. H., and Edward D. J. (1995). Disposition of intravenous and oral cyclosporin after administration with grapefruit juice. *Clinical Pharmacology and Therapeutics* 57(5): 485-491.

Eagling, V. A., Profit L., and Back D. J. (1999). Inhibition of the CYP3A4-mediated

metabolism and P-glycoprotein-mediated transport of the HIV-1 protease inhibitor saquinavir by grapefruit juice components. *British Journal of Clinical Pharmacology* 48: 543-552.

Edgar, B. , Bailey D. G., Bergstrand R., Johnsson G., and Lurje L. (1990). Formulation Dependent Interaction Between Felodipine and Grapefruit Juice. *Clinical Pharmacology & Therapeutics* 47(2): 181.

Edgar, B. , Bailey D., Bergstrand R., Johnsson G., and Regardh C. G. (1992). Acute Effects Of Drinking Grapefruit Juice On the Pharmacokinetics and Dynamics On Felodipine - and Its Potential Clinical Relevance. *European Journal Of Clinical Pharmacology* 42(3): 313-317.

Edwards, D. J. & Bernier S. M. (1996). Naringin and naringenin are not the primary CYP3A inhibitors in grapefruit juice. *Life Sciences* 59(13): 1025-1030.

Edwards, D. J. , Fitzsimmons M. E., Schuetz E. G., Yasuda K., Ducharme M. P., Warbasse L. H., Woster P. M., Schuetz J. D., and Watkins P. (1999). 6 ',7 '-Dihydroxybergamottin in grapefruit juice and Seville orange juice: Effects on cyclosporine disposition, enterocyte CYP3A4, and P-glycoprotein. *Clinical Pharmacology & Therapeutics* 65(3): 237-244.

Eichelbaum, M. , Ende M., Remberg G., Schomerus M., and Dengler H. J. (1979). The metabolism of DL-[14C]verapamil in man. *Drug Metabolism and Disposition* 7(3): 145-148.

Elegbede, J. A. , Elson C. E., Tanner M. A., Qureshi A., and Gould M. N. (1986). Regression Of Rat Primary Mammary-Tumors Following Dietary D- Limonene. *Journal Of the National Cancer Institute* 76(2): 323-325.

Eneroth, A., E. Astrom, J. Hoogstraate, D. Schrenk, S. Conrad, H. M. Kauffmann, and K. Gjellan (2001). Evaluation of a vincristine resistant Caco-2 cell line for use in a calcein AM extrusion screening assay for P-glycoprotein interaction. *European Journal Of Pharmaceutical Sciences* 12(3): 205-214.

Enjoh, M. , Hashimoto K., Arai S., and Shimizu M. (1996). Inhibitory effect of arphamenine A on intestinal dipeptide transport. *Bioscience Biotechnology and Biochemistry* 60(11): 1893-1895.

Essodaigui, M., Broxterman H. J., and Garnier-Suillerot A. (1998). Kinetic analysis of calcein and calcein-acetoxymethylester efflux mediated by the multidrug resistance protein and p-glycoprotein. *Biochemistry* 37: 2243-2250.

Eytan, G. D. , Regev R., and Assaraf Y. G. (1996). Functional reconstitution of P-glycoprotein reveals an apparent near stoichiometric drug transport to ATP hydrolysis. *Journal Of Biological Chemistry* 271(6): 3172-3178.

Fawcus, K. , Gorton V. J., Lucas M. L., and McEwan G. T. A. (1997). Stimulation of three distinct guanylate cyclases induces mucosal surface alkalinisation in rat small intestine in vitro. *Comparative Biochemistry and Physiology a-Physiology* 118(2): 291-295.

Fei, Y. , Kanal Y., Nussberger S., Ganapathy V., Leibach F. H., Romero M. F., Singh S. K., Boron W. F., and Hediger M. A. (1994). Expression cloning of a mammalian proton-coupled oligopeptide transporter. *Nature* 368: 563-566.

Feller, N., C. M. Kuiper, J. Lankelma, J. K. Ruhdal, R. J. Scheper, H. M. Pinedo, and H. J. Broxterman (1995). Functional Detection Of Mdr1/P170 and Mrp/P190-Mediated Multidrug-Resistance In Tumor-Cells By Flow-Cytometry. *British Journal Of Cancer* 72(3): 543-549.

Figgitt, D. P. & Plosker G. L. (2000). Saquinavir soft-gel capsule - An updated review of its use in the management of HIV infection. *Drugs* 60(2): 481-516.

Fogh, J. , Fogh J. M., and Orfeo T. (1977). One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *Journal of the National Cancer Institute, USA* 59(1): 221-226.

Fojo, A. T. , Ueda K., Slamon D. J., Poplack D. G., Gottesman M. M., and Pastan I. (1987). Expression of a multidrug-resistance gene in human tumors and tissues. *Proceedings of the National Academy of Sciences USA* 84(1): 265-269.

Formica, J. V. & Regelson W. (1995). Review of the biology of quercetin and related bioflavonoids. *Food and Chemical Toxicology* 33(12): 1061-1080.

Foster, B. C., Gallicano K. D., Cameron W., and Choudri S. H. (1998). Constituents of garlic inhibit cytochrome P-450 3A4 mediated drug metabolism. *Canadian Journal of Infectious Diseases* 9(B): 472.

Foster, B. C. , Foster M. S., Vandenhoek S., Krantis A., Budzinski J. W., Arnason J. T., Gallicano K. D., and Choudri S. (2001). An in vitro evaluation of human cytochrome P450 3A4 and P- glycoprotein inhibition by garlic. *Journal Of Pharmacy and Pharmaceutical Sciences* 4(2): 176-184.

Fuhr, U. , Klittich K., and Staib A. H. (1993). Inhibitory Effect Of Grapefruit Juice and Its Bitter Principal, Naringenin, On Cyp1a2 Dependent Metabolism Of Caffeine In Man. *British Journal Of Clinical Pharmacology* 35(4): 431-436.

Fuhr, U. , Maier A., Keller A., Steinijans V. W., Sauter R., and Staib A. H. (1995). Lacking Effect Of Grapefruit Juice On Theophylline Pharmacokinetics. *International Journal Of Clinical Pharmacology and Therapeutics* 33(6): 311-314.

Fuhr, U. , Maier-Bruggemann A., Blume H., Muck W., Unger S., Kuhlmann J., Huschka C., Zaigler M., Rietbrock S., and Staib A. H. (1998). Grapefruit juice increases oral nimodipine bioavailability. *International Journal Of Clinical Pharmacology and Therapeutics* 36(3): 126-132.

Fujita, T. , Morishita Y., Ito H., Kuribayashi D., Yamamoto A., and Muranishi S. (1997). Enhancement of the small intestinal uptake of phenylalanylglycine via a H+/oligopeptide transport system by chemical modification with fatty acids. *Life Sciences* 61(25): 2455-2465.

Fukutake, M. , Takahashi M., Ishida K., Kawamura H., Sugimura T., and Wakabayashi K. (1996). Quantification of genistein and genistin in soybeans and soybean products. *Food and Chemical Toxicology* 34(5): 457-461.

Gallicano, K. , Foster B., and Choudhri S. (2003). Effect of short-term administration of garlic supplements on single-dose ritonavir pharmacokinetics in healthy volunteers. *British Journal Of Clinical Pharmacology* 55(2): 199-202.

Garg, S. K. , Kumar N., Bhargava V. K., and Prabhakar S. K. (1998). Effect of grapefruit juice on carbamazepine bioavailability in patients with epilepsy. *Clinical Pharmacology & Therapeutics* 64(3): 286-288.

Garg, A. , Garg S., Zaneveld L. J. D., and Singla A. K. (2001). Chemistry and pharmacology of the Citrus bioflavonoid hesperidin. *Phytotherapy Research* 15(8): 655-669.

Gartiez, D.A., R.H. Hook, B.J. Walker, and R.A. Okerholm (1982). Pharmacokinetics and Biotransformation Studies of Terfenadine in Man. *Arzneimittel-Forschung* 32(9a): 1185-1190.

Gel-Moreto, N. , Streich R., and Galensa R. (2001). Chiral separation of six diastereomeric flavanone-7-O- glycosides by capillary electrophoresis and analysis of lemon juice. *Journal Of Chromatography a* 925(1-2): 279-289.

Gervot, L., Rochat B., Gautier J. C., Bohnenstengel F., Kroemer H., de Berardinis V., Martin H., Beaune P., and de Waziers I. (1999). Human CYP2B6: expression, inducibility and catalytic activities. *Pharmacogenetics* 9(3): 295-306.

Giorgi, M. , Salvatori A. P., Soldani G., Giusiani M., Longo V., Gervasi P. G., and Mengozzi G. (2001). Pharmacokinetics and microsomal oxidation of praziquantel and its effects on the P450 system in three-month-old lambs infested by Fasciola hepatica. *Journal Of Veterinary Pharmacology and Therapeutics* 24(4): 251-259.

Gollapudi, S. , Kim C. H., Tran B. N., Sangha S., and Gupta S. (1997). Probenecid reverses multidrug resistance in multidrug resistance-associated protein-overexpressing HL60/AR and H69/AR cells but not in P-glycoprotein-overexpressing HL60/Tax and P388/ADR cells. *Cancer Chemotherapy and Pharmacology* 40(2): 150-158.

Gottesman, M. M. & Pastan I. (1993). Biochemistry Of Multidrug-Resistance Mediated By

the Multidrug Transporter. *Annual Review Of Biochemistry* 62: 385-427.

Graf, E. (1992). Antioxidant Potential Of Ferulic Acid. *Free Radical Biology and Medicine* 13(4): 435-448.

Griffith, O.W. & A. Meister (1979). Potent and Specific Inhibition of Glutathione Synthesis by Buthionine Sulfoximine (s-n-Butyl Homocystein Sulfoximine). *The Journal of Biological Chemistry* 254(16): 7558-7560.

Gross, Annette S, Yan D Goh, Russell S Addison, and Gillian M Shenfield (1999). Influence of grapefruit juice on cisapride pharmacokinetics. *Clinical Pharmacology and Therapeutics* 65: 395-401.

Grunewald, K. K. & Bailey R. S. (1993). Commercially Marketed Supplements For Bodybuilding Athletes. *Sports Medicine* 15(2): 90-103.

Guengerich, F.P., Martin M.V., Beaune P.H., Kremers P., Wolff T., and Waxman D.J. (1986). Characterization Of Rat and Human-Liver Microsomal Cytochrome- P-450 Forms Involved In Nifedipine Oxidation, a Prototype For Genetic-Polymorphism In Oxidative Drug-Metabolism. *Journal Of Biological Chemistry* 261(11): 5051-5060.

Guengerich, F. P. & D. H. Kim (1990). Invitro Inhibition Of Dihydropyridine Oxidation and Aflatoxin- B1 Activation In Human Liver-Microsomes By Naringenin and Other Flavonoids. *Carcinogenesis* 11(12): 2275-2279.

Guo, A., Hu P., Balimane P. V., Leibach F. H., and Sinko P. J. (1999). Interactions of a nonpeptidic drug, valacyclovir, with the human intestinal peptide transporter (hPEPT1) expressed in a mammalian cell line. *Journal of Pharmacology and Experimental Therapeutics* 289: 448-454.

Gupta, M. C. , Garg S. K., Badyal D., Malhotra S., and Bhargava V. K. (1999). Effect of grapefruit juice on the pharmacokinetics of theophylline in healthy male volunteers. *Methods and Findings in Experimental and Clinical Pharmacology* 21(10): 679-682.

Haehner, B. D. , Gorski J. C., Vandenbranden M., Wrighton S. A., Janardan S. K., Watkins

P. B., and Hall S. D. (1996). Bimodal distribution of renal cytochrome P450 3A activity in humans. *Molecular Pharmacology* 50(1): 52-59.

Hait, W.N., J.F. Gesmonde, J.R. Murren, H. Ahmad, M. Reiss, and J-M Yang (1992). Terfenadine (Seldane(R)): A potent sensitizer of multidrug resistant cells. *Proceedings of the American Association for Cancer Research* 33: 479.

Hait, W. N., Gesmonde J.F., Murren J. R., Yang Jin-ming., Chen H. X., and Reiss M. (1993). Terfenadine (Seldane(R)) - a New Drug For Restoring Sensitivity to Multidrug Resistant Cancer-Cells. *Biochemical Pharmacology* 45(2): 401-406.

Han, H. , Oh D., and Amidon G. L. (1998). Cellular uptake mechanism of amino acid ester prodrugs in Caco-2/hPEPT1 cells overexpressing a human peptide transporter. *Pharmaceutical Research* 15(9): 1382-1386.

Hashimoto, K. , Shirafuji T., Sekino H., Matsuoka O., Sekino H., Onnagawa O., Okamoto T., Kudo S., and Azuma J. (1998). Interaction of citrus juices with pranidipine, a new 1,4- dihydropyridine calcium antagonist, in healthy subjects. *European Journal Of Clinical Pharmacology* 54(9-10): 753-760.

Hebert, M.F., Roberts J.P., Prueksaritanont T., and Benet L.Z. (1992). Bioavailability Of Cyclosporine With Concomitant Rifampin Administration Is Markedly Less Than Predicted By Hepatic Enzyme-Induction. *Clinical Pharmacology & Therapeutics* 52(5): 453-457.

Hellman, K. , Miller D. S., and Cammack K. A. (1983). The Effect Of Freeze-Drying On the Quaternary Structure Of L- Asparaginase From Erwinia-Carotovora. *Biochimica Et Biophysica Acta* 749(2): 133-142.

Hennessy, M., Kelleher D., Spiers J. P., Barry M., Kavanagh P., Back D., Mulcahy F., and Feely J. (2002). St John's Wort increases expression of P-glycoportein: Implications for drug interactions. *British Journal of Clinical Pharmacology* 53: 75-82.

Herlitz, H. , Edgar B., Hedner T., Lidman K., and Karlberg I. (1993). Grapefruit Juice - a Possible Source Of Variability In Blood- Concentration Of Cyclosporine-a.

Nephrology Dialysis Transplantation 8(4): 375.

Hertog, M. G. L., Hollman P. C. H., and Venema D. P. (1992). Optimization Of a Quantitative Hplc Determination Of Potentially Anticarcinogenic Flavonoids In Vegetables and Fruits. *Journal Of Agricultural and Food Chemistry* 40(9): 1591-1598.

Hertog, M.G.L., Hollman P.C.H., and Vandeputte B. (1993). Content Of Potentially Anticarcinogenic Flavonoids Of Tea Infusions, Wines, and Fruit Juices. *Journal Of Agricultural and Food Chemistry* 41(8): 1242-1246.

Herweijer, H. , Sonneveld P., Baas F., and Nooter K. (1990). Expression Of Mdr1 and Mdr3 Multidrug-Resistance Genes In Human Acute and Chronic Leukemias and Association With Stimulation Of Drug Accumulation By Cyclosporine. *Journal Of the National Cancer Institute* 82(13): 1133-1140.

Hidalgo, I. J., Bhatnagar P., Lee C., Miller J., Cucullino G., and Smith P. L. (1995). Structural requirements for interaction with the oligopeptide transporter in Caco-2 cells. *Pharmaceutical Research* 12(2): 317-319.

Higgins, C. F. & Gottesman M. M. (1992). Is the Multidrug Transporter a Flippase. *Trends In Biochemical Sciences* 17(1): 18-21.

Hirohashi, T., Suzuki H., Chu X., Tamai I., Tsuji A., and Sugiyama Y. (2000). Function and expression of multidrug resistance-associated protein family in human colon adenocarcinoma cells (Caco- 2). *Journal of Pharmacology and Experimental Therapeutics* 292(1): 265-270.

Ho, P. C. , Chalcroft S. C., Coville P. F., and Wanwimolruk S. (1999). Grapefruit juice has no effect on quinine pharmacokinetics. *European Journal Of Clinical Pharmacology* 55(5): 393-398.

Ho, P. C., Saville D. J., Coville P. F., and Wanwimolruk S. (2000). Content of CYP3A4 inhibitors, naringen, naringenin and bergapten in grapefruit juice products. *Pharmaceutica Acta Helvetiae* 74: 379-385.

Hofer, A. , Kerl H., and Wolf P. (2001). Long-term results in the treatment of vitiligo with oral khellin plus UVA. *European Journal Of Dermatology* 11(3): 225-229.

Holasova, M. , Fiedlerova V., Smrcinova H., Orsak M., Lachman J., and Vavreinova S. (2002). Buckwheat - the source of antioxidant activity in functional foods. *Food Research International* 35(2-3): 207-211.

Hollander, A. A. M. J., Vanrooij J., Lentjes E. G. W. M., Arbouw F., Vanbree J. B., Schoemaker R. C., Vanes L. A., Vanderwoude F. J., and Cohen A. F. (1995). The Effect Of Grapefruit Juice On Cyclosporine and Prednisone Metabolism In Transplant Patients. *Clinical Pharmacology & Therapeutics* 57(3): 318-324.

Hollo, Z. , Homolya L., Davis C. W., and Sarkadi B. (1994). Calcein Accumulation As a Fluorometric Functional Assay Of the Multidrug Transporter. *Biochimica Et Biophysica Acta-Biomembranes* 1191(2): 384-388.

Hollo, Z., Homolya L., Hegedus T., and Sarkadi B. (1996). Transport properties of the multidrug resistance-associated protein (MRP) in human tumour cells. *Febs Letters* 383(1-2): 99-104.

Homolya, L., Z. Hollo, U. A. Germann, I. Pastan, M. M. Gottesman, and B. Sarkadi (1993). Fluorescent Cellular Indicators Are Extruded By the Multidrug- Resistance Protein. *Journal Of Biological Chemistry* 268(29): 21493-21496.

Homolya, L., Z. Hollo, M. Muller, E. B. Mechetner, and B. Sarkadi (1996). A new method for quantitative assessment of P-glycoprotein- related multidrug resistance in tumour cells. *British Journal Of Cancer* 73(7): 849-855.

Honig, P. K. , Wortham D. C., Lazarev A., and Cantilena L. R. (1996). Grapefruit juice alters the systemic bioavailability and cardiac repolarization of terfenadine in poor metabolizers of terfenadine. *Journal Of Clinical Pharmacology* 36(4): 345-351.

Hopfer, U. , Nelson K., Perrotto J., and Isselbacher K. J. (1973). Glucose transport in isolated brush border membrane from rat small intestine. *Journal of Biological Chemistry* 248(1): 25-32.

Howell, S. , Kenny A. J., and Turner A. J. (1992). A Survey Of Membrane Peptidases In 2 Human Colonic Cell-Lines, Caco-2 and Ht-29. *Biochemical Journal* 284: 595-601.

Hu, M. & Amidon G. L. (1988). Passive and carrier-mediated intestinal absorption components of captopril. *Journal of Pharmaceutical Sciences* 77(12): 1007-1011.

Hu, M., Subramanian P., Mosberg H. I., and Amidon G. L. (1989). Use of the peptide carrier system to improve the intestinal absorption of L-a-methyldopa: Carrier kinetics, intestinal permeabilities, and in vitro hydrolysis of dipeptidyl derivatives of L-a-methyldopa. *Pharmaceutical Research* 6(1): 66-70.

Hu, M. , Chen J., Zhu Y., Dantzig A. H., Stratford R. E., and Kuhfeld M.T. (1994). Mechanism and kinetics of transcellular trransport of a new b-lactam antibiotic loracarbef across an intestinal epithelial membrane model system (Caco-2). *Pharmaceutical Research* 11(10): 1405-1412.

Hu, M., Y. Q. Li, C. M. Davitt, S. M. Huang, K. Thummel, B. W. Penman, and C. L. Crespi (1999). Transport and metabolic characterization of Caco-2 cells expressing CYP3A4 and CYP3A4 plus oxidoreductase. *Pharmaceutical Research* 16(9): 1352-1359.

Hudson, E. A. , Dinh P. A., Kokubun T., Simmonds M. S. J., and Gescher A. (2000). Characterization of potentially chemopreventive phenols in extracts of brown rice that inhibit the growth of human breast and colon cancer cells. *Cancer Epidemiology Biomarkers & Prevention* 9(11): 1163-1170.

Hukkinen, S. K., Varhe A., Olkkola K. T., and Neuvonen P. J. (1995). Plasma-Concentrations Of Triazolam Are Increased By Concomitant Ingestion Of Grapefruit Juice. *Clinical Pharmacology & Therapeutics* 58(2): 127-131.

Ikegawa, T. , Ushigome F., Koyabu N., Morimoto S., Shoyama Y., Naito M., Tsuruo T., Ohtani H., and Sawada Y. (2000). Inhibition of P-glycoprotein by orange juice components, polymethoxyflavones in adriamycin-resistant human myelogenous leukemia (K562/ADM) cells. *Cancer Letters* 160(1): 21-28.

Ioannides-Demos, L. L., Christophidis N., Ryan P., Angelis P., Liolios L., and McLean A. J. (1997). Dosing implications of a clinical interaction between grapefruit juice and cyclosporine and metabolite concentrations in patients with autoimmune diseases. *Journal of Rheumatology* 24(1): 49-54.

Jackson, G. M., Hall D. E., and Walker R. (1987). Comparison Of the Short-Term Hepatic-Effects Of Orally- Administered Citral In Long Evans Hooded and Wistar Albino-Rats. *Food and Chemical Toxicology* 25(7): 505-513.

Jacquemin, E. , Hagenbuch B., Stieger B., Wolkoff A. W., and Meier P. J. (1994). Expression Cloning Of a Rat-Liver Na+-Independent Organic Anion Transporter. *Proceedings Of the National Academy Of Sciences Of the United States Of America* 91(1): 133-137.

Jonsson, B., G. Liminga, K. Csoka, H. Fridborg, S. Dhar, P. Nygren, and R. Larsson (1996). Cytotoxic activity of calcein acetoxymethyl ester (Calcein/AM) on primary cultures of human haematological and solid tumors. *European Journal Of Cancer* 32A(5): 883-887.

Josefsson, M. , Zackrisson A. L., and Ahlner J. (1996). Effect of grapefruit juice on the pharmacokinetics of amlodipine in healthy volunteers. *European Journal of Clinical Pharmacology* 51(2): 189-193.

Kakemi, K. , Arita T., Hori R., Konishi R., and Nishimura K. (1969). Absorption and exretion of drugs. XXXIV. An aspect of the mechanism of drug absorption from the intestinal tract in rats. *Chemistry and Pharmacy Bulletin (Tokyo)* 17(2): 255-261.

Kantola, T. , Kivişto K. T., and Neuvonen P. J. (1998). Grapefruit juice greatly increases serum concentrations of lovastatin and lovastatin acid. *Clinical Pharmacology & Therapeutics* 63(4): 397-402.

Kararli, T. T. (1989). Gastrointestinal absorption of drugs. *Critical Reviews in Therapeutic Drug Carrier Systems* 6: 39-86.

Katoh, M. , Nakajima M., Yamazaki H., and Yokoi T. (2000). Inhibitory potencies of 1,4-dihydropyridine calcium antagonists to p-glycoprotein-mediated transport: Comparison with the effects of CYP3A4. *Pharmaceutical Research* 17(10): 1189-1197.

Katoh, M. , Nakajima M., Yamazaki H., and Yokoi T. (2001). Inhibitory effects of CYP3A4 substrates and their metabolites on P-glycoprotein-mediated transport. *European Journal Of Pharmaceutical Sciences* 12(4): 505-513.

Kawakami, M. , Suzuki K., Ishizuka T., Hidaka T., Matsuki Y., and Nakamura H. (1998). Effect of grapefruit juice on pharmacokinetics of itraconazole in healthy subjects. *International Journal Of Clinical Pharmacology and Therapeutics* 36(6): 306-308.

Kearns, G. L. , Lowry J., Khan I., Kashuba A., Bertino J., Nafziger A., Abdel-Rahman S., and Leeder J. (2002). Cisapride (CIS): A potential model substrate to assess CYP3A4 activity. *Clinical Pharmacology & Therapeutics* 71(2): WPIII-104.

Kikuzaki, H. , Hisamoto M., Hirose K., Akiyama K., and Taniguchi H. (2002). Antioxidant properties of ferulic acid and its related compounds. *Journal Of Agricultural and Food Chemistry* 50(7): 2161-2168.

Kim, R. B. , Wandel C., Leake B., Cvetkovic M., Fromm M. F., Dempsey P. J., Roden M. M., Belas F., Chaudhary A. K., Roden D. M., Wood A. J. J., and Wilkinson G. R. (1999). Interrelationship between substrates and inhibitors of human CYP3A and P-glycoprotein. *Pharmaceutical Research* 16(3): 408-414.

Kirbaslar, F. G. , Kirbaslar S. I., and Dramur U. (2001). The compositions of Turkish bergamot oils produced by cold- pressing and steam distillation. *Journal Of Essential Oil Research* 13(6): 411-415.

Kivisto, K. T. , Lamberg T. S., Kantola T., and Neuvonen P. J. (1997). Plasma buspirone concentrations are greatly increased by erythromycin and itraconazole. *Clinical Pharmacology & Therapeutics* 62(3): 348-354.

Kivisto, K. T. , Lilja T. J., Backman J. T., and Neuvonen P. J. (1999). Repeated consumption of grapefruit juice considerably increases plasma concentrations of

cisapride. *Clinical Pharmacology & Therapeutics* 66(5): 448-453.

Kolars, J. C., Awni W. M., Merion R. M., and Watkins P. B. (1991). 1st-Pass Metabolism Of Cyclosporine By the Gut. *Lancet* 338(8781): 1488-1490.

Kolars, J. C., Schmiedlinren P., Schuetz J. D., Fang C., and Watkins P. B. (1992). Identification Of Rifampin-Inducible P450iiia4 (Cyp3a4) In Human Small-Bowel Enterocytes. *Journal Of Clinical Investigation* 90(5): 1871-1878.

Ku, Y. M. , Min D. I., and Flanigan M. (1998). Effect of grapefruit juice on the pharmacokinetics of microemulsion cyclosporine and its metabolite in healthy volunteers: Does the formulation difference matter? *Journal Of Clinical Pharmacology* 38(10): 959-965.

Kudo, S. , Okumura H., Miyamoto G., and Ishizaki T. (1999). Cytochrome P-450 isoforms involved in carboxylic acid ester cleavage of Hantzsch pyridine ester of pranidipine. *Drug Metabolism and Disposition* 27(2): 303-308.

Kumar, N. , Garg S. K., and Prabhakar S. (1999). Lack of pharmacokinetic interaction between grapefruit juice and phenytoin in healthy male volunteers and epileptic patients. *Methods and Findings in Experimental and Clinical Pharmacology* 21(9): 629-632.

Kupferschmidt, H. H. T., Ha H. R., Ziegler W. H., Meier P. J., and Krahenbuhl S. (1995). Interaction Between Grapefruit Juice and Midazolam In Humans. *Clinical Pharmacology & Therapeutics* 58(1): 20-28.

Kupferschmidt, H. H. T. , Fattinger K. E., Ha H. R., Follath F., and Krahenbuhl S. (1998). Grapefruit juice enhances the bioavailability of the HIV protease inhibitor saquinavir in man. *British Journal Of Clinical Pharmacology* 45(4): 355-359.

Kwak, M. K., Kim S. G., and Kim N. D. (1995). Effects Of Garlic Oil On Rat Hepatic P4502e1 Expression. *Xenobiotica* 25(10): 1021-1029.

Laroche, M. , Choudhri S., Gallicano K., and Foster B. (1998). Severe gas-trointestinal

toxicity with concomitant ingestion of ritonavir and garlic. *Canadian Journal of Infectious Diseases* 9: P471.

Lecureur, V., D. X. Sun, P. Hargrove, E. G. Schuetz, R. B. Kim, L. B. Lan, and J. D. Schuetz (2000). Cloning and expression of murine sister of P-glycoprotein reveals a more discriminating transporter than MDR1/P- glycoprotein. *Molecular Pharmacology* 57(1): 24-35.

Lee, C. G. L. , Ramachandra M., Jeang K. T., Martin M. A., Pastan I., and Gottesman M. M. (2000). Effect of ABC transporters on HIV-1 infection: inhibition of virus production by the MDR1 transporter. *Faseb Journal* 14(3): 516-522.

Leeson, T. S., Leeson C. R., and Paparo A. A. (1988). Atlas of Histology. Philadelphia, W B Saunders.

Legrand, O., Simonin G., Perrot J., Zittoun R., and Marie J. (1998). Pgp and MRP activites using calcein-AM are prognostic factors in adult acute myeloid leukemia patients. *Blood* 91(12): 4480-4488.

Leier, I. , Jedlitschky G., Buchholz U., Cole S. P. C., Deeley R. G., and Keppler D. (1994). The Mrp Gene Encodes an Atp-Dependent Export Pump For Leukotriene C-4, and Structurally Related Conjugates. *Journal Of Biological Chemistry* 269(45): 27807-27810.

Leonard, J. V. , Marrs T. C., Addison J. M., Burston D, Clegg K. M., Lloyd J. K., Matthews D. M., and Seakins J. W. (1976). Intestinal absorption of amino acids and peptides in Hartnup disorder. *Pediatric Research* 10(4): 246-249.

Li, L. Q. , Lee T. K., Meier P. J., and Ballatori N. (1998). Identification of glutathione as a driving force and leukotriene C-4 as a substrate for oatp1, the hepatic sinusoidal organic solute transporter. *Journal Of Biological Chemistry* 273(26): 16184-16191.

Liang, R., Fei Y., Prasad P. D., Ramamoorthy S., Han H., Yang-Feng T. L., Hediger M. A., Ganapathy V., and Leibach F. H. (1995). Human intestinal H^+/peptide cotransporter: Cloning, functional expression, and chromosomal localization. *The*

Journal of Biological Chemistry 270(12): 6456-6463.

Lilja, J. J. , Kivisto K. T., and Neuvonen P. J. (1998a). Grapefruit juice-simvastatin interaction: Effect on serum concentrations of simvastatin, simvastatin acid, and HMG-CoA reductase inhibitors. *Clinical Pharmacology & Therapeutics* 64(5): 477-483.

Lilja, J. J. , Kivisto K. T., Backman J. T., Lamberg T. S., and Neuvonen P. J. (1998b). Grapefruit juice substantially increases plasma concentrations of buspirone. *Clinical Pharmacology & Therapeutics* 64(6): 655-660.

Lilja, J. J. , Kivisto K. T., and Neuvonen P. J. (1999). Grapefruit juice increases serum concentrations of atorvastatin and has no effect on pravastatin. *Clinical Pharmacology & Therapeutics* 66(2): 118-127.

Lilja, J. J. , Kivisto K. T., Backman J. T., and Neuvonen P. J. (2000a). Effect of grapefruit juice dose on grapefruit juice-triazolam interaction: repeated consumption prolongs triazolam half-life. *European Journal Of Clinical Pharmacology* 56(5): 411-415.

Lilja, J. J. , Kivisto K. T., and Neuvonen P. J. (2000b). Duration of effect of grapefruit juice on the pharmacokinetics of the CYP3A4 substrate simvastatin. *Clinical Pharmacology & Therapeutics* 68(4): 384-390.

Liminga, G., Nygren P., and Larsson R. (1994). Microfluorometric evaluation of calcein acetoxymethyl ester as a probe for P-glycoprotein-mediated resistance: Effects of Cyclosporin A and its nonimmunosuppressive analogue SDZ PSC 833. *Experimental Cell Research* 212: 291-296.

Liminga, G. , Nygren P., Dhar S., Nilsson K., and Larsson R. (1995). Cytotoxic Effect Of Calcein Acetoxymethyl Ester On Human Tumor- Cell Lines - Drug-Delivery By Intracellular Trapping. *Anti-Cancer Drugs* 6(4): 578-585.

Liu, R. H. & Sharom F. J. (1996). Site-directed fluorescence labeling of P-glycoprotein on cysteine residues in the nucleotide binding domains. *Biochemistry* 35(36): 11865-11873.

Loe, D. W. , Oleschuk C. J., Deeley R. G., and Cole S. P. C. (2000). Structure-activity studies of verapamil analogs that modulate transport of leukotriene C-4 and reduced glutathione by multidrug resistance protein MRP1. *Biochemical and Biophysical Research Communications* 275(3): 795-803.

Lown, K. S. , Kolars J. C., Thummel K. E., Barnett J. L., Kunze K. L., Wrighton S. A., and Watkins P. B. (1994). Interpatient Heterogeneity In Expression Of Cyp3a4 and Cyp3a5 In Small-Bowel - Lack Of Prediction By the Erythromycin Breath Test. *Drug Metabolism and Disposition* 22(6): 947-955.

Lown, K. S. , Kolars J. C., Ghosh M., Schmiedlin-Ren P., and Watkins P. B. (1996). Induction of MDR1 expression in normal rat and human intestine in vivo. *Gastroenterology* 110(4): A344.

Lown, K. S., Bailey D. G., Fontana R. J., Janardan S. K., Adair C. H., Fortlage L. A., Brown M. B., Guo W., and Watkins P. B. (1997). Grapefruit juice increases felodipine oral availability in humans by decreasing intestinal CYP3A protein expression. *Journal of Clinical Investigation* 99: 2545-2553.

Lundahl, J., Regardh C. G., Edgar B., and Johnsson G. (1995). Relationship between time of intake of grapefruit juice and its effect on pharmacokinetics and pharmacodynamics of felodipine in healthy subjects. *1995* 49: 61-67.

Lundahl, J. , Regardh C. G., Edgar B., and Johnsson G. (1997). Effects of grapefruit juice ingestion - Pharmacokinetics and haemodynamics of intravenously and orally administered felodipine in healthy men. *European Journal Of Clinical Pharmacology* 52(2): 139-145.

Lundahl, J. U. E. , Regardh C. G., Edgar B., and Johnsson G. (1998). The interaction effect of grapefruit juice is maximal after the first glass. *European Journal Of Clinical Pharmacology* 54(1): 75-81.

Lutzky, J. , Astor M. B., Taub R. N., Baker M. A., Bhalla K., Gervasoni J. E., Rosado M., Stewart V., Krishna S., and Hindenburg A. A. (1989). Role Of Glutathione and Dependent Enzymes In Anthracycline- Resistant Hl60/Ar Cells. *Cancer Research*

49(15): 4120-4125.

Ma, B. , Prueksaritanont T., and Lin J. H. (2000). Drug interactions with calcium channel blockers: Possible involvement of metabolite-intermediate complexation with CYP3A. *Drug Metabolism and Disposition* 28(2): 125-130.

Maish, W. A. , Hampton E. M., Whitsett T. L., Shepard J. D., and Lovallo W. R. (1996). Influence of grapefruit juice on caffeine pharmacokinetics and pharmacodynamics. *Pharmacotherapy* 16(6): 1046-1052.

Malhotra, S. , Bailey D. G., Paine M. F., and Watkins P. B. (2001). Seville orange juice-felodipine interaction: Comparison with dilute grapefruit juice and involvement of furocoumarins. *Clinical Pharmacology & Therapeutics* 69(1): 14-23.

Manthey, J. A. & Grohmann K. (2001). Distributions of flavonoids and hydroxycinnamates in orange fruit. *Abstracts Of Papers Of the American Chemical Society* 221: 67.

Matheny, C. J. , Lamb M. W., Brouwer K. L. R., and Pollack G. M. (2001). Pharmacokinetic and pharmacodynamic implications of P- glycoprotein modulation. *Pharmacotherapy* 21(7): 778-796.

Matthews, D. M. (1975). Intestinal absorption of peptides. *Physiological Reviews* 55(4): 537-608.

McKinnon, R. A. , Burgess W. M., Hall P. D., Abdulaziz Z., and Mcmanus M. E. (1992). Metabolism Of Food-Derived Heterocyclic Amines In Human and Rabbit-Tissues By P4503a Proteins In the Presence Of Flavonoids. *Cancer Research* 52(7): S2108-S2113.

Meijer, C., Mulder N. H., Timmer-Bosscha H., Peters W. H. M., and de Vries E. G. E. (1991). Combined *In Vitro* Modulation of Adriamycin Resistance. *International Journal of Cancer* 49(4): 582-586.

Melzig, M. F. , Loose R., and Schonherr G. (1997). Effect of flavonoids on daunomycin-induced toxicity in cultivated endothelial cells. *Pharmazie* 52(10): 793-796.

Meredith, D. , Boyd C. A. R., Bronk J. R., Bailey P. D., Morgan K. M., Collier I. D., and Temple C. S. (1998). 4-aminomethylbenzoic acid is a non-translocated competitive inhibitor of the epithelial peptide transporter PepT1. *Journal Of Physiology-London* 512(3): 629-634.

Merry, C. , Barry M. G., Mulcahy F., Ryan M., Heavey J., Tjia J. F., Gibbons S. E., Breckenridge A. M., and Back D. J. (1997). Saquinavir pharmacokinetics alone and in combination with ritonavir in HIV-infected patients. *Aids* 11(4): F29-F33.

Meunier, V., Bourrie M., Berger Y., and Fabre G. (1995). The human intestinal epithelial cell line Caco-2; pharmacological and pharmacokinetic applications. *Cell Biology and Toxicology* 11: 187-194.

Mikus, G. , Eichelbaum M., Fischer C., Gumulka S., Klotz U., and Kroemer H.K. (1990). Interaction Of Verapamil and Cimetidine - Stereochemical Aspects Of Drug-Metabolism, Drug Disposition and Drug-Action. *Journal Of Pharmacology and Experimental Therapeutics* 253(3): 1042-1048.

Min, D. I. , Ku Y. M., Perry P. J., Ukah F. O., Ashton K., Martin M. F., and Hunsicker L. G. (1996a). Effect of grapefruit juice on cyclosporine pharmacokinetics in renal transplant patients. *Transplantation* 62(1): 123-125.

Min, D. I. , Ku Y. M., Geraets D. R., and Lee H. C. (1996b). Effect of grapefruit juice on the pharmacokinetics and pharmacodynamics of quinidine in healthy volunteers. *Journal Of Clinical Pharmacology* 36(5): 469-476.

Minta, A. , Kao J. P. Y., and Tsien R. Y. (1989). Fluorescent Indicators For Cytosolic Calcium Based On Rhodamine and Fluorescein Chromophores. *Journal Of Biological Chemistry* 264(14): 8171-8178.

Mitsunaga, Y., H. Takanaga, H. Matsuo, M. Naito, T. Tsuruo, H. Ohtani, and Y. Sawada (2000). Effect of bioflavonoids on vincristine transport across blood- brain barrier. *European Journal Of Pharmacology* 395(3): 193-201.

Miyake, Y. , Yamamoto K., Tsujihara N., and Osawa T. (1998). Protective effects of lemon flavonoids on oxidative stress in diabetic rats. *Lipids* 33(7): 689-695.

Moore, V. A., W. J. Irwin, P. Timmins, S. Chong, S. A. Dando, and R. A. Morrison (2000). A rapid screening system to determine drug affinities for the intestinal dipeptide transporter 1: system characterisation. *International Journal Of Pharmaceutics* 210(1-2): 15-27.

Mooseker, M. S. & Tilney L. G. (1975). Organization of an actin filament-membrane complex. Filament polarity and membrane attachment in the microvilli of intestinal epithelial cells. *Journal of Cell Biology* 67(3): 725-743.

Morgan, S. A., Watson J. V., Twentyman P. R., and Smith P. J. (1989). Flow Cytometric Analysis Of Hoechst-33342 Uptake As an Indicator Of Multi-Drug Resistance In Human-Lung Cancer. *British Journal Of Cancer* 60(3): 282-287.

Muller, M. , Meijer C., Mulder N. H., Devries E. G. E., and Jansen P. L. M. (1994). Is the Multispecific Organic Anion Transporter (Moat) the Multidrug-Resistance Related Protein (Mrp). *Hepatology* 20(4): A173.

Neuvonen, P. J. , Lilja J., and Kivisto K. T. (1999). Grapefruit juice increases serum simvastatin and atorvastatin but not pravastatin. *Clinical Pharmacology & Therapeutics* 65(2): PIII13.

Nicklin, P. L., Irwin W. J., Hassan I. F., and Mackay M. (1992). Proline Uptake By Monolayers Of Human Intestinal Absorptive (Caco-2) Cells-Invitro. *Biochimica Et Biophysica Acta* 1104(2): 283-292.

Oelkers, B. , Diehl H., and Liebig H. (1992). Invitro Inhibition Of Cytochrome-P-450 Reductases From Pig- Liver Microsomes By Garlic Extracts. *Arzneimittel-Forschung/Drug Research* 42(1): 136-139.

Ohtani, M. , Kawabata S., Kariya S., Uchino K., Itou K., Kotaki H., Kasuyama K., Morikawa A., Seo I., and Nishida N. (2002). Effect of grapefruit pulp on the pharmacokinetics of the dihydropyridine calcium antagonists nifedipine and

nisoldipine. *Yakugaku Zasshi-Journal Of the Pharmaceutical Society Of Japan* 122(5): 323-329.

Ozdemir, M. , Aktan Y., Boydag B. S., Cingi M. I., and Musmul A. (1998). Interaction between grapefruit juice and diazepam in humans. *European Journal Of Drug Metabolism and Pharmacokinetics* 23(1): 55-59.

Paine, M. F. , Khalighi M., Fisher J. M., Shen D. D., Kunze K. L., Marsh C. L., Perkins J. D., and Thummel K. E. (1997). Characterization of interintestinal and intraintestinal variations in human CYP3A-dependent metabolism. *Journal Of Pharmacology and Experimental Therapeutics* 283(3): 1552-1562.

Parkinson, A. (1996). An overview of current cytochrome P450 technology for assessing the safety and efficacy of new materials. *Toxicologic Pathology* 24(1): 45-57.

Pascaud, C. , Garrigos M., and Orlowski S. (1998). Multidrug resistance transporter P-glycoprotein has distinct but interacting binding sites for cytotoxic drugs and reversing agents. *Biochemical Journal* 333: 351-358.

Paul, S. , Breuninger L. M., Tew K. D., Shen H. X., and Kruh G. D. (1996). ATP-dependent uptake of natural product cytotoxic drugs by membrane vesicles establishes MRP as a broad specificity transporter. *Proceedings Of the National Academy Of Sciences Of the United States Of America* 93(14): 6929-6934.

Pauli-Magnus, C., O. von Richter, O. Burk, A. Ziegler, T. Mettang, M. Eichelbaum, and M. F. Fromm (2000). Characterization of the major metabolites of verapamil as substrates and inhibitors of P-glycoprotein. *Journal Of Pharmacology and Experimental Therapeutics* 293(2): 376-382.

Pawagi, A. B., Wang J., Silverman M., Reithmeier R. A. F., and Deber C. M. (1994). Transmembrane Aromatic Amino-Acid Distribution In P- Glycoprotein - a Functional-Role In Broad Substrate-Specificity. *Journal Of Molecular Biology* 235(2): 554-564.

Pearce, H. L., Safa A. R., Bach N. J., Winter M. A., Cirtain M. C., and Beck W. T. (1989). Essential Features Of the P-Glycoprotein Pharmacophore As Defined By a Series Of

Reserpine Analogs That Modulate Multidrug Resistance. *Proceedings Of the National Academy Of Sciences Of the United States Of America* 86(13): 5128-5132.

Penzak, S. R. , Gubbins P. O., Gurley B. J., Wang P. L., and Saccente M. (1999). Grapefruit juice decreases the systemic availability of itraconazole capsules in healthy volunteers. *Therapeutic Drug Monitoring* 21(3): 304-309.

Phang, J. M., Poore C. M., Lopaczynska J., and Yeh G. C. (1993). Flavonol-Stimulated Efflux Of 7,12-Dimethylbenz(a)Anthracene In Multidrug-Resistant Breast-Cancer Cells. *Cancer Research* 53(24): 5977-5981.

Phang, J. M., C. M. Poore, J. Lopaczynska, and G. C. Yeh (1994). Kaempferol stimulates the Pgp-mediated efflux of adriamycin (ADR) and dimethylbenzanthracene (DMBA) in multi-drug resistant MCF-7 cells by a redox-dependent mechanism. *Proceedings of the American Association for Cancer Research* 35: 344.

Pietta, P. , Mauri P., Bruno A., Rava A., Manera E., and Ceva P. (1991). Identification Of Flavonoids From Ginkgo-Biloba L, Anthemis- Nobilis L and Equisetum-Arvense L By High-Performance Liquid- Chromatography With Diode-Array Uv Detection. *Journal Of Chromatography* 553(1-2): 223-231.

Pino, J. A. & Sanchez M. (2000). Chemical composition of grapefruit oil concentrates. *Journal Of Essential Oil Research* 12(2): 167-169.

Proppe, D. G., Hoch O. D., Mclean A. J., and Visser K. E. (1995). Influence Of Chronic Ingestion Of Grapefruit Juice On Steady- State Blood-Concentrations Of Cyclosporine-a In Renal- Transplant Patients With Stable Graft Function. *British Journal Of Clinical Pharmacology* 39(3): 337-338.

Proppe, D. , Visser K., Bartels R., Hoch O., Meyer H., and McLean A. J. (1996). Grapefruit juice selectively modifies cyclosporin A metabolite patterns in renal transplant patients. *Clinical Pharmacology & Therapeutics* 59(2): PI35.

Raeissi, S. D. , Hidalgo I. J., Segura-Aguilar J., and Artursson P. (1999). Interplay between CYP3A-mediated metabolism and polarized efflux of terfenadine and its metabolites in

intestinal epithelial Caco-2 (TC7) cell monolayers. *Pharmaceutical Research* 16(5): 625-632.

Ramu, A. , Spanier R., Rahamimoff H., and Fuks Z. (1984). Restoration Of Doxorubicin Responsiveness In Doxorubicin- Resistant P388 Murine Leukemia-Cells. *British Journal Of Cancer* 50(4): 501-507.

Rashid, J. , Mckinstry C., Renwick A. G., Dirnhuber M., Waller D. G., and George C. F. (1993). Quercetin, an In-Vitro Inhibitor Of Cyp3a, Does Not Contribute to the Interaction Between Nifedipine and Grapefruit Juice. *British Journal Of Clinical Pharmacology* 36(5): 460-463.

Rashid, T. J., Martin U., Clarke H., Waller D. G., Renwick A. G., and George C. F. (1995). Factors Affecting the Absolute Bioavailability Of Nifedipine. *British Journal Of Clinical Pharmacology* 40(1): 51-58.

Rau, S. E. , Bend J. R., Arnold J. M. O., Tran L. T., Spence J. D., and Bailey D. G. (1997). Grapefruit juice terfenadine single-dose interaction: Magnitude, mechanism, and relevance. *Clinical Pharmacology & Therapeutics* 61(4): 401-409.

Roby, C. A. , Anderson G. D., Kantor E., Dryer D. A., and Burstein A. H. (2000). St John's Wort: Effect on CYP3A4 activity. *Clinical Pharmacology & Therapeutics* 67(5): 451-457.

Roffey, S. J., Walker R., and Gibson G. G. (1990). Hepatic Peroxisomal and Microsomal-Enzyme Induction By Citral and Linalool In Rats. *Food and Chemical Toxicology* 28(6): 403-408.

Rogers, J. D. , Vega J. M., Zhao J., Amin R. D., Gagliano K., Stepanavage M., Wilson M. F., and Blum R. A. (1999a). The effect of grapefruit juice on plasma profiles of lovastatin, lovastatin acid, and lovastatin HMG-CoA reductase inhibitors. *Atherosclerosis* 144: 183.

Rogers, J. D. , Vega J. M., Zhao J., Liu L., Amin R. D., Gagliano K., Stepanavage M., Wilson M. F., and Blum R. A. (1999b). Grapefruit juice (GFJ) has a small effect on

lovastatin plasma HMG-CoA reductase inhibitor (HMGRI) profiles. *Clinical Pharmacology & Therapeutics* 65(2): PII10.

Rukmini, C. & Raghuram T. C. (1991). Nutritional and Biochemical Aspects Of the Hypolipidemic Action Of Rice Bran Oil - a Review. *Journal Of the American College Of Nutrition* 10(6): 593-601.

Rundlof, T. , Olsson E., Wiernik A., Back S., Aune M., Johansson L., and Wahlberg I. (2000). Potential nitrite scavengers as inhibitors of the formation of N-nitrosamines in solution and tobacco matrix systems. *Journal Of Agricultural and Food Chemistry* 48(9): 4381-4388.

Russo, A. , Acquaviva R., Campisi A., Sorrenti V., Di Giacomo C., Virgata G., Barcellona M. L., and Vanella A. (2000). Bioflavonoids as antiradicals, antioxidants and DNA cleavage protectors. *Cell Biology and Toxicology* 16(2): 91-98.

Saeki, T. , Ueda K., Tanigawara Y., Hori R., and Komano T. (1993). Human P-Glycoprotein Transports Cyclosporine-a and Fk506. *Journal Of Biological Chemistry* 268(9): 6077-6080.

Sahu, S. C. & Gray G. C. (1994). Kaempferol-Induced Nuclear-Dna Damage and Lipid-Peroxidation. *Cancer Letters* 85(2): 159-164.

Saito, H. & Inui K. (1993). Dipeptide transporters in apical and basolateral membranes of the human intestinal cell line Caco-2. *American Journal of Physiology* 265: G289-G294.

Satlin, L. M. , Amin V., and Wolkoff A. W. (1997). Organic anion transporting polypeptide mediates organic anion/HCO3- exchange. *Journal Of Biological Chemistry* 272(42): 26340-26345.

Scambia, G. , Ranelletti F. O., Panici P. B., Devincenzo R., Bonanno G., Ferrandina G., Piantelli M., Bussa S., Rumi C., Cianfriglia M., and Mancuso S. (1994). Quercetin Potentiates the Effect Of Adriamycin In a Multidrug- Resistant Mcf-7 Human Breast-Cancer Cell-Line - P-Glycoprotein As a Possible Target. *Cancer Chemotherapy and*

Pharmacology 34(6): 459-464.

Schlatter, J. , Zimmerli B., Dick R., Panizzon R., and Schlatter C. (1991). Dietary-Intake and Risk Assessment Of Phototoxic Furocoumarins In Humans. *Food and Chemical Toxicology* 29(8): 523-530.

Schmiedlin-Ren, P., Thummel K. E., Fisher J. M., Paine M. F., Lown K. S., and Watkins P. B. (1997). Expression of enzymatically active CYP3A4 by Caco-2 cells grown on extracellular matrix-coated permeable supports in the presence of 1a,25-dihydroxyvitamin D_3. *The Journal of Pharmacology and Experimental Therapeutics* 51: 741-754.

Schoenmakers, R. G. , Stehouwer M. C., and Tukker J. J. (1999). Structure-transport relationship for the intestinal small- peptide carrier: Is the carbonyl group of the peptide bond relevant for transport? *Pharmaceutical Research* 16(1): 62-68.

Schubert, W., Cullberg G., Edgar B., and Hedner T. (1994). Inhibition of 17 beta-estradiol metabolism by grapefruit juice in ovariectomized women. *Maturitas* 20(2-3): 155-163.

Schuetz, E. G., Schuetz J. D., Grogan W. M., Narayfejestoth A., Fejestoth G., Raucy J., Guzelian P., Gionela K., and Watlington C. O. (1992). Expression Of Cytochrome-P450 3a In Amphibian, Rat, and Human Kidney. *Archives Of Biochemistry and Biophysics* 294(1): 206-214.

Schuetz, J. D., Beach D. L., and Guzelian P. S. (1994). Selective Expression Of Cytochrome-P450 Cyp3a Messenger-Rnas In Embryonic and Adult Human Liver. *Pharmacogenetics* 4(1): 11-20.

Shanson, D. C., Mcnabb R., and Hajipieris P. (1984). The Effect Of Probenecid On Serum Amoxycillin Concentrations Up to 18 Hours After a Single 3 G Oral Dose Of Amoxycillin - Possible Implications For Preventing Endocarditis. *Journal Of Antimicrobial Chemotherapy* 13(6): 629-632.

Shapiro, A. B. & Ling V. (1995). Reconstitution Of Drug Transport By Purified P-Glycoprotein. *Journal Of Biological Chemistry* 270(27): 16167-16175.

Shapiro, A. B. & Ling V. (1997). Positively cooperative sites for drug transport by P-glycoprotein with distinct drug specificities. *European Journal Of Biochemistry* 250(1): 130-137.

Sharom, F. J. (1997). The P-glycoprotein efflux pump: How does it transport drugs? *Journal Of Membrane Biology* 160(3): 161-175.

Shaw, P. E. , Goodner K. L., Moshonas M. G., and Hearn C. J. (2001). Comparison of grapefruit hybrid fruit with parent fruit based on composition of volatile components. *Scientia Horticulturae* 91(1-2): 71-80.

Shelton, M. J. , Wynn H. E., Newitt R. G., and DiFrancesco R. (2001). Effects of grapefruit juice on pharmacokinetic exposure to indinavir in HIV-positive subjects. *Journal Of Clinical Pharmacology* 41(4): 435-442.

Shimada, T. , Gillam E. M. J., Sandhu P., Guo Z. Y., Tukey R. H., and Guengerich F. P. (1994). Activation Of Procarcinogens By Human Cytochrome-P450 Enzymes Expressed In Escherichia-Coli - Simplified Bacterial Systems For Genotoxicity Assays. *Carcinogenesis* 15(11): 2523-2529.

Sigusch, H. , Henschel L., Kraul H., Merkel U., and Hoffmann A. (1994). Lack Of Effect Of Grapefruit Juice On Diltiazem Bioavailability In Normal Subjects. *Pharmazie* 49(9): 675-679.

Sinko, P. J. & Amidon G. L. (1989). Characterisation of the oral absorption of B-lactam antibiotics II: Competitive absorption and peptide carrier specificity. *Journal of Pharmaceutical Sciences* 78(9): 723-727.

Sloley, B. D. , Urichuk L. J., Morley P., Durkin J., Shan J. J., Pang P. K. T., and Coutts R. T. (2000). Identification of kaempferol as a monoamine oxidase inhibitor and potential neuroprotectant in extracts of Ginkgo biloba leaves. *Journal Of Pharmacy and Pharmacology* 52(4): 451-459.

Smith, P. L., Wall D. A., Gochoco C. H., and Wilson G. (1992). (D) Routes of delivery:

Case Studies (5) Oral absorption of peptides and proteins. *Advanced Drug Delivery Reviews* 8: 253-290.

Solomon, P. R. , Adams F., Silver A., Zimmer J., and DeVeaux R. (2002). Ginkgo for memory enhancement - A randomized controlled trial. *Jama-Journal Of the American Medical Association* 288(7): 835-840.

Soons, P. A., Vogels B. A. P. M., Roosemalen M. C. M., Schoemaker H. C., Uchida E., Edgar B., Lundahl J., Cohen A. F., and Breimer D. D. (1991). Grapefruit Juice and Cimetidine Inhibit Stereoselective Metabolism Of Nitrendipine In Humans. *Clinical Pharmacology & Therapeutics* 50(4): 394-403.

Spatzenegger, M. & Jaeger W. (1995). Clinical Importance Of Hepatic Cytochrome-P450 In Drug- Metabolism. *Drug Metabolism Reviews* 27(3): 397-417.

Stremple, P. (1998). GC/MS analysis of polymethoxyflavones in citrus oils. *Hrc-Journal Of High Resolution Chromatography* 21(11): 587-591.

Stride, B. D. , Valdimarsson G., Gerlach J. H., Wilson G. M., Cole S. P. C., and Deeley R. G. (1996). Structure and expression of the messenger RNA encoding the murine multidrug resistance protein, an ATP-binding cassette transporter. *Molecular Pharmacology* 49(6): 962-971.

Swaan, P. W. & Tukker J. J. (1997). Molecular determinants of recognition for the intestinal peptide carrier. *Journal of Pharmaceutical Sciences* 86(5): 596-602.

Takanaga, H. , Ohnishi A., Murakami H., Matsuo H., Higuchi S., Urae A., Irie S., Furuie H., Matsukuma K., Kimura M., Kawano K., Orii Y., Tanaka T., and Sawada Y. (2000). Relationship between time after intake of grapefruit juice and the effect on pharmacokinetics and pharmacodynamics of nisoldipine in healthy subjects. *Clinical Pharmacology & Therapeutics* 67(3): 201-214.

Tamai, I. & Safa A. R. (1990). Competitive Interaction Of Cyclosporins With the Vinca Alkaloid-Binding Site Of P-Glycoprotein In Multidrug-Resistant Cells. *Journal Of Biological Chemistry* 265(27): 16509-16513.

Tamai, I. & Tsuji A. (1996). Drug delivery through the blood-brain barrier. *Advanced Drug Delivery Reviews* 19: 401-424.

Tamura, K., Bhatnagar P. K., Takata J. S., Lee C., Smith P. L., and Borchardt R. T. (1996). Metabolism, uptake, and transepithelial transport of the diastereoisomers of val-val in the human intestinal cell line, Caco-2. *Pharmaceutical Research* 13(8): 1213-1218.

Tateoka, R. , Abe H., Miyauchi S., Shuto S., Matsuda A., Kobayashi M., Miyazaki K., and Kamo N. (2001). Significance of substrate hydrophobicity for recognition by an oligopeptide transporter (PEPT1). *Bioconjugate Chemistry* 12(4): 485-492.

Taub, M. E., Due Larsen B. D., Steffansen B., and Frokjaer S. (1997a). b-Carboxylic acid esterified D-Asp-Ala retains a high affinity for the oligopeptide transporter in Caco-2 monolayers. *International Journal of Pharmaceutics* 146: 205-212.

Taub, M. E., Moss B. A., Steffansen B., and Frokjaer S. (1997b). Influence of oligopeptide transporter binding affinity upon uptake and transport of D-Asp(OBzl)-Ala and Asp(OBzl)-Sar in filter-grown Caco-2 monolayers. *International Journal of Pharmaceutics* 156: 219-228.

Temple, C. S., Stewart A. K., Meredith D., Lister N. A., Morgan K. M., Ian D Collier I. D., Vaughan-Jones R. D., Boyd C. A. R., Bailey P. D., and Bronk J. R. (1998). Peptide mimics as substrates for the intestinal peptide transporter. *The Journal of Biological Chemistry* 273(1): 20-22.

Thummel, K. E. & Wilkinson G. R. (1998). In vitro and in vivo drug interactions involving human CYP3A. *Annual Review Of Pharmacology and Toxicology* 38: 389-430.

Thwaites, D. T., Brown C. D. A., Hirst B. H., and Simmons N. L. (1993). Transepithelial glycylsarcosine transport in intestinal Caco-2 cells mediated by expression of H^+-coupled carriers at both apical and basal membranes. *The Journal of Biological Chemistry* 268(11): 7640-7642.

Tirona, R. G. & Kim R. B. (2002). Pharmacogenomics of organic anion-transporting polypeptides (OATP). *Advanced Drug Delivery Reviews* 54(10): 1343-1352.

Tsien, R.Y. (1983). Intracellular measurement of ion activities. *Annual Review of Biophysics and Bioengineering* 12: 91-116.

Tsuji, A., Tamai I., Nakanishi M., Terasaki T., and Hamano S. (1993). Intestinal brush-border transport of the oral cephalosporin antibiotic, cefdinir, mediated by dipeptide and monocarboxylic acid transport systems in rabbits. *Journal of Pharmacy and Pharmacology* 45: 996-998.

Tsuji, A. & Tamai I. (1996). Carrier-mediated intestinal transport of drugs. *Pharmaceutical Research* 13(7): 963-977.

Tsuruo, T. , Iida H., Yamashiro M., Tsukagoshi S., and Sakurai Y. (1982). Enhancement Of Vincristine-Induced and Adriamycin-Induced Cyto- Toxicity By Verapamil In P388 Leukemia and Its Sublines Resistant to Vincristine and Adriamycin. *Biochemical Pharmacology* 31(19): 3138-3140.

Twentyman, P. R. , Rhodes T., and Rayner S. (1994). A Comparison Of Rhodamine-123 Accumulation and Efflux In Cells With P-Glycoprotein-Mediated and Mrp-Associated Multidrug- Resistance Phenotypes. *European Journal Of Cancer* 30A(9): 1360-1369.

Ubeda, A. & Villar A. (1989). Relaxant Actions Of Khellin On Vascular Smooth-Muscle. *Journal Of Pharmacy and Pharmacology* 41(4): 236-241.

Ueda, K. , Taguchi Y., and Morishima M. (1997). How does P-glycoprotein recognize its substrates? *Seminars In Cancer Biology* 8(3): 151-159.

Uno, T. , Ohkubo T., Sugawara K., Higashiyama A., and Motomura S. (1997). Effect of grapefruit juice on the disposition of nicardipine after administration of intravenous and oral doses. *Clinical Pharmacology & Therapeutics* 61(2): PII60.

Uno, T. , Ohkubo T., Sugawara K., Higashiyama A., Motomura S., and Ishizaki T. (2000). Effects of grapefruit juice on the stereoselective disposition of nicardipine in humans:

evidence for dominant presystemic elimination at the gut site. *European Journal Of Clinical Pharmacology* 56(9-10): 643-649.

van Agtmael, M. A. , Gupta V., van der Wosten T. H., Rutten J. P. B., and van Boxtel C. J. (1999). Grapefruit juice increases the bioavailability of artemether. *European Journal Of Clinical Pharmacology* 55(5): 405-410.

van Gelder, J. , Shafiee M., De Clercq E., Penninckx F., van den Mooter G., Kinget R., and Augustijns P. (2000). Species-dependent and site-specific intestinal metabolism of ester prodrugs. *International Journal Of Pharmaceutics* 205(1-2): 93-100.

Varis, T. , Kivisto K. T., and Neuvonen P. J. (2000). Grapefruit juice can increase the plasma concentrations of oral methylprednisolone. *European Journal Of Clinical Pharmacology* 56(6-7): 489-493.

Versantvoort, C. H. M., Broxterman H. J., Lankelma J., Feller N., and Pinedo H. M. (1994). Competitive-Inhibition By Genistein and Atp Dependence Of Daunorubicin Transport In Intact Mrp Overexpressing Human Small-Cell Lung-Cancer Cells. *Biochemical Pharmacology* 48(6): 1129-1136.

Versantvoort, C. H. M., Broxterman H. J., Bagrij T., Scheper R. J., and Twentyman P. R. (1995). Regulation By Glutathione Of Drug Transport In Multidrug- Resistant Human Lung-Tumor Cell-Lines Overexpressing Multidrug Resistance-Associated Protein. *British Journal Of Cancer* 72(1): 82-89.

Versantvoort, C. H. M. , Rhodes T., and Twentyman P. R. (1996). Acceleration of MRP-associated efflux of rhodamine 123 by genistein and related compounds. *British Journal Of Cancer* 74(12): 1949-1954.

Vincent, J. , Foulds G., Dogolo L. C., Willavize S. A., and Friedman H. L. (1997). Grapefruit juice does not alter the pharmacokinetics of amlodipine in man. *Clinical Pharmacology & Therapeutics* 61(2): PIV70.

Vincent, J. , Harris S. I., Foulds G., Dogolo L. C., Willavize S., and Friedman H. L. (2000). Lack of effect of grapefruit juice on the pharmacokinetics and

pharmacodynamics of amlodipine. *British Journal Of Clinical Pharmacology* 50(5): 455-463.

Wacher, V. J., Wu C. Y., and Benet L. Z. (1995). Overlapping Substrate Specificities and Tissue Distribution Of Cytochrome-P450 3a and P-Glycoprotein - Implications For Drug- Delivery and Activity In Cancer-Chemotherapy. *Molecular Carcinogenesis* 13(3): 129-134.

Wall, D. M., Hu X. F., Zalcberg J. R., and Parkin J. D. (1991). Rapid Functional Assay For Multidrug Resistance In Human Tumor- Cell Lines Using the Fluorescent Indicator Fluo-3. *Journal Of the National Cancer Institute* 83(3): 206-207.

Wallach, D. F. H. & Steck T. L. (1963). Fluorescence techniques in the microdetermination of metals in biological materials: Utility of 2,4-bis-[N,N'-di-(carboxymethyl)aminomethyl] fluorescein in the fluorometric estimation of Al+3, alkaline earths, Co+2, Cu+2, Ni+2, and Zn+2 in micromolar concentrations. *Analytical Chemistry* 35(8): 1035-1044.

Walter, E., Kissel T., and Amidon G. L. (1996a). The intestinal peptide carrier: A potential transport system for small peptide derived drugs. *Advanced Drug Delivery Reviews* 20: 33-58.

Walter, E., Janich S., Roessler B. J., Hilfinger J. M., and Amidon G. L. (1996b). HT29-MTX/Caco-2 cocultures as an in vitro model for the intestinal epithelium: In vitro - in vivo correlation with permeability data from rats and humans. *Journal of Pharmaceutical Sciences* 85(10): 1070-1076.

Wang, E. J. , Casciano C. N., Clement R. P., and Johnson W. W. (2001a). HMG-CoA reductase inhibitors (statins) characterized as direct inhibitors of P-glycoprotein. *Pharmaceutical Research* 18(6): 800806.

Wang, Z. Q. , Gorski C., Hamman M. A., Huang S. M., Lesko L. J., and Hall S. D. (2001b). The effects of St John's wort (*Hypericum perforatum*) on human cytochrome P450 activity. *Clinical Pharmacology & Therapeutics* 70(4): 317-326.

Watabe, T. , Hiratsuka A., Ozawa N., and Isobe M. (1981). A Comparative-Study On the Metabolism Of D-Limonene and 4- Vinylcyclohex-1-Ene By Hepatic Microsomes. *Xenobiotica* 11(5): 333-344.

Watkins, P. B., Wrighton S. A., Schuetz E. G., Molowa D. T., and Guzelian P. S. (1987). Identification Of Glucocorticoid-Inducible Cytochromes-P-450 In the Intestinal-Mucosa Of Rats and Man. *Journal Of Clinical Investigation* 80(4): 1029-1036.

Watkins, P. B. (1994). Noninvasive Tests Of Cyp3a Enzymes. *Pharmacogenetics* 4(4): 171-184.

Wattenberg, L. W. & Coccia J. B. (1991). Inhibition Of 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-Butanone Carcinogenesis In Mice By D-Limonene and Citrus-Fruit Oils. *Carcinogenesis* 12(1): 115-117.

Weaver, J. L., Pine P. S., Aszalos A., Schoenlein P. V., Currier S. J., Padmanabhan R., and Gottesman M. M (1991). Laser Scanning and Confocal Microscopy Of Daunorubicin, Doxorubicin, and Rhodamine-123 In Multidrug-Resistant Cells. *Experimental Cell Research* 196(2): 323-329.

Weber, A. , Jager R., Borner A., Klinger G., Vollanth R., Matthey K., and Balogh A. (1996). Can grapefruit juice influence ethinylestradiol bioavailability? *Contraception* 53(1): 41-47.

Weber, A. , Kaplan M., Chughtai S. A., Cohn L. A., Smith A. L., and Unadkat J. D. (2001). CYP3A inductive potential of the rifamycins, rifabutin and rifampin, in the rabbit. *Biopharmaceutics & Drug Disposition* 22(4): 157-168.

Wilson, F.A. & Dietschy J. M. (1972). Characterization of bile acid absorption across the unstirred water layer and brush border of the rat jejunum. *Journal of Clinical Investigation* 51(12): 3015-3025.

Woosley, R. L., Chen Y. W., Freiman J. P., and Gillis R. A. (1993). Mechanism Of the Cardiotoxic Actions Of Terfenadine. *Jama-Journal Of the American Medical Association* 269(12): 1532-1536.

Wootton, R. & Hazelwood R. (1989). Relative Affinity Of a Series Of Charged Dipeptides For the Peptide Carrier Of Rabbit Intestinal Brush-Border Membranes. *Biochemical Society Transactions* 17(4): 691-692.

Wrighton, S. A., Ring B. J., Watkins P. B., and Vandenbranden M. (1989). Identification Of a Polymorphically Expressed Member Of the Human Cytochrome P-450iii Family. *Molecular Pharmacology* 36(1): 97-105.

Wrighton, S. A., Brian W. R., Sari M. A., Iwasaki M., Guengerich F. P., Raucy J. L., Molowa D. T., and Vandenbranden M. (1990). Studies On the Expression and Metabolic Capabilities Of Human Liver Cytochrome P450iiia5 (Hlp3). *Molecular Pharmacology* 38(2): 207-213.

Wu, C., Benet L. Z., Hebert M. F., Gupta S. K., Rowland M., Gomez D. Y., and Wacher V. J. (1995). Differentiation of absorption and first-pass gut and hepatic metabolism in humans: Studies with cyclosporine. *Clinical Pharmacology and Therapeutics* 58: 492-497.

Yamada, K. , Murakami M., Yamamoto A., Takada K., and Muranishi S. (1992). Improvement Of Intestinal-Absorption Of Thyrotropin-Releasing- Hormone By Chemical Modification With Lauric Acid. *Journal Of Pharmacy and Pharmacology* 44(9): 717-721.

Yamada, J. & Tomita Y. (1996). Antimutagenic activity of caffeic acid and related compounds. *Bioscience Biotechnology and Biochemistry* 60(2): 328-329.

Yee, G. C., Stanley D. L., Pessa L. J., Costa T. D., Beltz S. E., Ruiz J., and Lowenthal D. T. (1995). Effect Of Grapefruit Juice On Blood Cyclosporine Concentration. *Lancet* 345(8955): 955-956.

Yoshimura, A. , Shudo N., Ikeda S., Ichikawa M., Sumizawa T., and Akiyama S.-I. (1990). Novel Screening Method For Agents That Overcome Classical Multidrug Resistance In a Human Cell-Line. *Cancer Letters* 50(1): 45-51.

Yu, H., Cook T. J., and Sinko P. J. (1997). Evidence for diminished functional expression of intestinal tranporters in Caco-2 cell monolayers at high passages. *Pharmaceutical Research* 14(6): 757-762.

Zaidenstein, R. , Dishi V., Gips M., Soback S., Cohen N., Weissgarten J., Blatt A., and Golik A. (1998). The effect of grapefruit juice on the pharmacokinetics of orally administered verapamil. *European Journal Of Clinical Pharmacology* 54(4): 337-340.

Zaidenstein, R. , Avni B., Dishi V., Gipps M., Soback S., Koren M., Simantov R., and Golik A. (1999). Effect of grapefruit juice on the pharmacokinetics of losartan in healthy volunteers. *Clinical Pharmacology & Therapeutics* 65(2): PI60.

Zaidenstein, R. , Soback S., Gips M., Avni B., Dishi V., Weissgarten Y., Golik A., and Scapa E. (2001). Effect of grapefruit juice on the pharmacokinetics of losartan and its active metabolite E3174 in healthy volunteers. *Therapeutic Drug Monitoring* 23(4): 369-373.

APPENDIX 1. MICHAELIS-MENTON KINETICS

A1.1 Calculation of kinetic parameters

The binding of a transport protein to its substrate resembles that of an enzyme-substrate reaction. When the transporter protein is saturated, the rate of uptake will be maximal; this rate is referred to as V_{max}. The transporter has a characteristic binding constant for the substrate. This Michaelis constant is referred to as K_m, which is equivalent to the concentration of substrate for half maximal uptake. The relationship of the reaction is shown in Equation A2.1 below.

Equation A1.1 The Michaelis-Menton Equation for Active Uptake

$$V = \frac{(V_{max} \times [S])}{(K_m + [S])}$$

Where:

V = Uptake (moles/mg protein/minute)

V_{max} = Maximum uptake (moles/mg protein/minute)

$[S]$ = Initial substrate concentration (moles/litre)

K_m = Michaelis constant (moles/litre)

Measured uptake will include a passive component. The Michaelis-Menten equation can be adapted to include a new constant related to passive uptake, K_d. This adapted equation is shown below.

Equation A1.2 The Adapted Michaelis-Menton Equation for Total Uptake

$$V = \left(\frac{(V_{max} \times [S])}{(K_m + [S])} \right) + (K_d \times [S])$$

Where:

V = Uptake (moles/mg protein/minute)

V_{max} = Maximum uptake (moles/mg protein/minute)

$[S]$ = Initial substrate concentration (moles/litre)

K_m = Michaelis constant (moles/litre)

K_d = Passive uptake constant (moles/mg protein/minute/(moles/litre))

Computer software can be used to calculate K_d. The passive component of uptake, once known, can be removed only leaving active uptake. Plotting active uptake at different concentrations (V against [S]) produces a curved line that flattens off. It is not easy to calculate V_{max} or K_m from this curve so different plots are used. Inverting Equation A1.1 produces the following one (Equation A1.3).

Equation A1.3 Inverting the Michaelis-Menton Equation

$$\left(\frac{1}{V}\right)=\left(\left(\frac{K_m}{V_{max}}\right)\times\left(\frac{1}{[S]}\right)\right)+\left(\frac{1}{V_{max}}\right)$$

Where:

V = Uptake (moles/mg protein/minute)

V_{max} = Maximum uptake (moles/mg protein/minute)

$[S]$ = Initial substrate concentration (moles/litre)

K_m = Michaelis constant (moles/litre)

Plotting $\left(\frac{1}{V}\right)$ against $\left(\frac{1}{[S]}\right)$ will produce a straight line with a gradient of $\left(\frac{K_m}{V_{max}}\right)$ and a y-intercept of $\left(\frac{1}{V_{max}}\right)$. This is known as a Lineweaver-Burk plot.

The Lineweaver-Burk plot derived from Equation A1.3 above can be used to calculate K_m and V_{max} although it is not very accurate. Inverting results at low concentrations produces large numbers so any errors are amplified while results are high concentrations are less emphasised. A more accurate plot is an Eadie-Hofstee plot derived from a rearranged Michaelis-Menten equation as shown in Equation A1.4 below. This plot produces a line with a gradient of $-K_m$ and a Y-intercept of V_{max}. Both kinetic parameters can be calculated and, as there is no inverting, errors are constant along the line.

Equation A1.4 Rearranging the Michaelis-Menton Equation

$$V = \frac{(V_{max} \times [S])}{(K_m + [S])}$$

Multiply by $(K_m + [S])$ $\quad V(K_m + [S]) = (V_{max} \times [S])$

Subtract $(V \times K_m)$ $\quad (V \times [S]) = (V_{max} \times [S]) - (V \times K_m)$

Divide by $[S]$ $\quad V = V_{max} - \left(K_m \times \left(\frac{V}{[S]}\right)\right)$

Where:

V = Uptake (moles/mg protein/minute)

V_{max} = Maximum uptake (moles/mg protein/minute)

$[S]$ = Initial substrate concentration (moles/litre)

K_m = Michaelis constant (moles/litre)

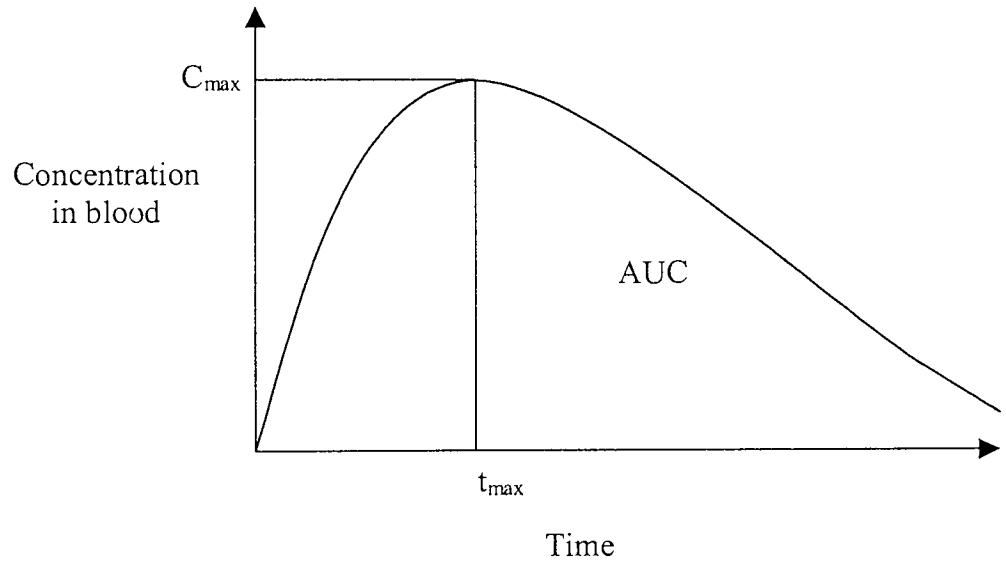
Plotting V against $\left(\frac{V}{[S]}\right)$ will produce a straight line with a gradient of $(-K_m)$ and a y-intercept of V_{max}. This is known as an Eadie-Hofstee plot.

APPENDIX 2. CLINICAL STUDIES WITH GRAPEFRUIT JUICE

A2.1 Pharmacokinetic parameters calculated during clinical studies

A clinical study of a drug measures the concentration of drug in the blood over time after oral absorption. A number of parameters can be calculated from the concentration-time plot. These include the area under the curve (AUC), the maximum concentration reached (C_{max}) and the time at which the maximum concentration was reached (t_{max}) (as shown in Figure A2.1 below).

Figure A2.1 Pharmacokinetic parameters calculated from clinical studies



A2.2 Clinical studies on grapefruit juice

The following table summarises every clinical study of the effect of grapefruit juice on the kinetics of co-administered drugs.

Dose of Drug (mg)	Route	Volume of GJ (ml)	Time of GJ (hr)	Study Group	Mean change as % control AUC	Cmax	tmax	Significance of change AUC	Cmax	tmax	Reference
17a-ethinylestradiol											
50ug	Oral	100	-0.5, 0	13w	+30.42	+37.65	+0.00	↑ *	↑ **	ns	Weber et al. (1996)
17b-estradiol											
2	Oral	200	0	8w	+16.00	+31.00	-23.00				Schubert et al. (1994)
Amlodipine											
10	IV	240	24 hourly (0-192)	20m	+4.47	-13.51	NA	ns	ns		Vincent et al. (1997 & 2000)
5	Oral	250	0	12	+13.83	+14.81	+8.57	↑ *	↑ *	ns	Joseffson et al. (1996)
10	Oral	240	24 hourly (0-192)	20m	+7.51	+6.90	-3.80	ns	ns	ns	Vincent et al. (1997 & 2000)
Artemether											
100	Oral	Glass	0	6	+89.83	+154.76	-41.67	↑ **	↑ ***	↓ *	van Agtmael et al. (1999)
Atorvastatin											
40	Oral	200	8 hourly (-48-0), 0.5, 1.5, 24, 32, 48, 56, 64, 72	12	+146.47	+5.51	+200.00	↑ **	ns	↑ **	Lilja et al. (1999) & Neuvonen et al. (1999)
Buspirone											
10	Oral	400	8 hourly (-48-0), 0.5, 1.5	10	+821.30	+328.57	+300.00	↑ **	↑ **	↑ **	Lilja et al. (1998b)
Caffeine											
3.3 /kg	Oral	200	0	10	+4.00	NA	NA				Maish et al. (1996)
3.3 /kg	Oral	200	-15, -9, -2, 0, 2, 6	6	+6.00	NA	NA				Maish et al. (1996)
165	Oral	300	-0.5, 6, 12, 18, 24, 30	12 (4m)	+28.00	NA	NA				Fuhr et al. (1993)
Carbamazepine											
200	Oral	300	0	10 epilepsy	+40.83	+40.46	-3.13	↑ **	↑ **	ns	Garg et al. (1998)
Cisapride											
10	Oral	250	0	14	+38.76	+33.85	+36.51	↑ **	↑ **	↑ *	Gross et al. (1999)
10	Oral	400	8 hourly (-48-0), 0.5, 1.5	10	+143.11	+81.25	+66.67	↑ **	↑ **	↑ *	Kivisto et al. (1999)

Dose of Drug (mg)	Route	Volume of GJ (ml)	Time of GJ (hr)	Study Group	Mean change as % control AUC	Cmax	tmax	Significance of change AUC	Cmax	tmax	Reference
Clarithromycin											
500	Oral	240	0	12 (6m)	+14.96	+4.17	+80.00	ns	ns	↑ *	Cheng et al. (1998)
Cyclosporin											
7.5 /kg	IV	250	0, 2	10,	+7.16	+2.88	NA	ns	ns		Ducharme et al. (1995)
1.7 /kg bid	Oral	150	12 hourly (-144 - 0)	9 (5m)	+42.85	+25.05	NA	↑ **	ns		Ioannides-Demos et al. (1997)
1.8 /kg bid	Oral	175	12 hourly (-144 - 0)	10 (5m)	+34.00	+10.00	+0.00				Proppe et al. (1995)
2.3-6.25/kg	Oral			10	+72.13	NA	NA	↑ **			Proppe et al. (1996)
5	Oral	6 ounces	0, 0.5, 1.5	12 (8m)	+47.43	+10.48	+23.53	↑ ***	↑ *	ns	Ku et al. (1998)
7.5 /kg	Oral	250	0, 2	10	+59.62	+43.16	+31.25	↑ *	↑ *	↑ *	Ducharme et al. (1995)
7.5 /kg	Oral	8 ounces	-0.5, 3.5, 7.5, 11.5	7 (5m)	+54.93	+35.35	NA	↑ *	↑ *		Edwards et al. (1999)
113 bid	Oral	200	0	6 RTP	+19.00	+27.00	NA				Herlitz et al. (1993)
175 bid	Oral	232	0	10 RTP	+23.51	+3.67	+92.86	↑ *	ns	↑ *	Min et al. (1996a)
300	Oral	250	0	14 (8m)	+43.09	+17.50	+21.19	↑ ***	↑ **	ns	Yee et al. (1995)
125-500	Oral	150	3 hourly (-7.5 - +22.5)	12 (8m) RTP	+7.59	+22.37	-22.89	ns	↑ **	ns	Hollander et al. (1995)
Diazepam											
5	Oral	250	0	8 (5m)	+224.07	+53.93	+37.33	↑ ***	↑ *	↑ **	Ozdemir et al. (1998)
Digoxin											
0.5	Oral	220	-0.5, 0, 3.5, 7.5, 11.5	12 (7m)	+9.58	+22.70	-7.69	↑ **	ns	ns	Becquemont et al. (2001)
Diltiazem											
120	Oral	200	0, 2, 4, 8, 12	9	+10.00	+2.00	+4.00				Sigush et al. (1994)

Dose of Drug (mg)	Route	Volume of GJ (ml)	Time of GJ (hr)	Study Group	Mean change as % control AUC	Cmax	tmax	Significance of change AUC	Cmax	tmax	Reference
Felodipine											
1.5	IV	150	-0.25	12m	-8.78	-11.66	NA	ns	ns		Lundahl et al. (1997)
5	Oral	200	0	9	+185.00	+146.90	+62.00	↑ **	↑ **	ns	Edgar et al. (1992)
5	Oral	400	0	9	+234.00	+191.00	+54.00	↑ **	↑ **	ns	Edgar et al. (1992)
5	Oral	200	0	9	+86.30	+75.00	+0.00	↑ **	↑ *	ns	Bailey et al. (1993)
5	Oral	500	0	6	+151.22	+123.08	+90.91	↑ **	↑ **	↑ **	Bailey et al. (1990 & 1991)
5	Oral	250	0	12 elderly	+187.50	+300.00	NA	↑ ***	↑ ***		Dresser et al. (2000)
5	Oral	200	0	7	+199.16	+167.19	NA	↑ ***	↑ **		Edgar et al. (1990)
5 SR	Oral	200	0	7	+80.88	+154.05	NA	↑ **	↑ ***		Edgar et al. (1990)
10	Oral	232	0	10	+116.43	+225.43	NA	↑ ***	↑ ***		Lown et al. (1997)
10	Oral	232	8 hourly (-120-+16)	10	+211.05	+334.54	NA	↑ ***	↑ ***		Lown et al. (1997)
10	Oral		0	12m	+73.00	+138.00					Lundahl et al. (1998)
10	Oral		24 hourly (0 - 336)	12m	+57.00	+114.00					Lundahl et al. (1998)
10 SR	Oral	200	0	9	+43.46	+92.39	-29.41	↑ ***	↑ ***	ns	Lundahl et al. (1995)
10 SR	Oral	200	-1	9	+45.52	+99.91	-35.29	↑ ***	↑ ***	ns	Lundahl et al. (1995)
10 SR	Oral	200	-4	9	+42.08	+54.35	-17.64	↑ ***	↑ **	ns	Lundahl et al. (1995)
10 SR	Oral	200	-10	9	+25.27	+54.35	+2.94	↑ *	↑ **	ns	Lundahl et al. (1995)
10 SR	Oral	200	-24	9	+12.68	+31.52	+0.00	ns	↑ *	ns	Lundahl et al. (1995)
10 SR	Oral	250	0	12	+92.60	+145.45	-20.00	↑ ***	↑ ***	ns	Bailey et al. (1994a & 1996)
10 SR	Oral	250	0	12 (10m)	+153.70	+128.57	-31.00	↑ ***	↑ ***	↓ **	Bailey et al. (2000a)
10 SR	Oral	240	0	10	+93.52	+87.84	+0.00	↑ *	↑ *	ns	Malhotra et al. (2001)
10 SR	Oral	150	-0.25	12m	+72.16	+173.13	-27.50	↑ **	↑ **	ns	Lundahl et al. (1997)
10 SR	Oral	250	0	12m	+101.42	+145.45	-6.67				Bailey et al. (1995)
10 SR	Oral	250	0	12m	+145.28	+200.00	-16.13	↑ ***	↑ ***	ns	Bailey et al. (1998a & 1998b)
20 SR	Oral	250	0	12	+119.00	+155.00	-17.00				Bailey et al. (1995)
Fexofenadine											
120	Oral	75	0	10 (6m)	-23.23	-20.83	+8.33	↓ *	ns	ns	Dresser et al. (2002)
120	Oral	300	0	10 (6m)	-66.99	-61.81	+33.33	↓ **	↓ **	ns	Dresser et al. (2002)
Indinavir											
800	Oral	180	0	14	-1.60	-16.77	+39.29	ns	ns	↑ *	Shelton et al. (2001)

Dose of Drug (mg)	Route	Volume of GJ (ml)	Time of GJ (hr)	Study Group	Mean change as % control AUC	Cmax	tmax	Significance of change AUC	Cmax	tmax	Reference
Itraconazole											
	Oral		0	11	-42.80		+37.50				Penzak et al. (1999)
100	Oral	350	0	22m	+3.32	-3.54	+4.26	ns	ns	ns	Kawakami et al. (1998)
Losartan											
	Oral	200	-1, 0	9	+14.20	NA	NA	ns			Zaidenstein et al. (1999) & Zaidenstein et al. (2001)
Lovastatin											
40	Oral	8 ounce	-49, -25, -1	16	+40.00	+40.00	NA	↑ **	↑ **		Rogers et al. (1999a)
40	Oral	250	24 hourly (-60 - -12), 0	16	+91.30	+125.58	-32.26	↑ ***	↑ ***	ns	Rogers et al. (1999b)
80	Oral	400	8 hourly (-48-0), 0.5, 1.5	10	+1,426.69	+1,077.14	+50.00	↑ ***	↑ ***	ns	Kantola et al. (1998)
Methylprednisolone											
16	Oral	200	8 hourly (-48-0), 0.5, 1.5	10	+72.18	+26.73	+50.00	↑ ***	↑ **	↑ *	Varis et al. (2000)
Midazolam											
5	IV	200	-1, -0.25	8m	+4.02	NA	NA	ns			Kupferschmidt et al. (1995)
2	Oral	8 ounce	-49, -25, -1	16	+139.13	+127.27	-16.67	↑ ***	↑ ***	ns	Rogers et al. (1999b)
15	Oral	200	-1, -0.25	8m	+51.75	+55.80	+79.03	↑ **	↑ **	↑ *	Kupferschmidt et al. (1995)
Nicardipine											
2 (+)	IV	300	-0.5	6m	-6.09	NA	NA	ns			Uno et al. (1997 & 2000)
2 (-)	IV	300	-0.5	6m	-4.33	NA	NA	ns			Uno et al. (1997 & 2000)
40 (+)	Oral	300	-0.5	6m	+43.50	NA	NA	↑ *			Uno et al. (1997 & 2000)
40 (-)	Oral	300	-0.5	6m	+84.20	NA	NA	↑ *			Uno et al. (1997 & 2000)

Dose of Drug (mg)	Route	Volume of GJ (ml)	Time of GJ (hr)	Study Group	Mean change as % control AUC	Cmax	tmax	Significance of change AUC	Cmax	tmax	Reference
Nifedipine											
2.5	IV	400	-2, 0	8 (5m)	+14.00	NA	NA				Rashid et al. (1995)
	Oral	Pulp	-1	8m	+31.49	+42.14	-48.40	↑ *	↑ *	ns	Ohtani et al. (2002)
	Oral	Pulp	1	8m	+10.82	+19.29	+0.00	ns	ns	ns	Ohtani et al. (2002)
10	Oral	500	0	6	+35.13	+12.61	+50.00	↑ *	ns	↑ *	Bailey et al. (1990 & 1991)
10	Oral	400	-2, 0	8 (5m)	+58.00	+16.00	+97.00				Rashid et al. (1995)
10	Oral	200	0	8	+46.79		+200.00				Rashid et al. (1993)
10	Oral		0, 8, 16	16 (8m)	+10.00			ns			Burggraaf et al. (1997)
20	Oral	250	-1	8m	-1.72	+31.05	+0.00	ns	ns	ns	Azuma et al. (1998)
20	Oral	250	0	8m	+8.75	+26.71	+50.00	ns	ns	↑ ***	Azuma et al. (1998)
20	Oral	250	1	8m	+13.33	+21.54	+100.00	ns	ns	↑ ***	Azuma et al. (1998)
20 SR	Oral	200	0, 2, 4, 8, 12	12	+103.00	+94.00	+73.00				Sigush et al. (1994)
60	GITS		0, 8, 16	16 (8m)	+10.00			ns			Burggraaf et al. (1997)
Nimodipine											
30	Oral	250	0	8	+51.34	+23.53	+112.50	↑ ***	↑ ***	↑ **	Fuhr et al. (1998)
Nisoldipine											
	Oral	Pulp	-1	8m	+28.94	+50.47	-36.93	↑ *	ns	ns	Ohtani et al. (2002)
	Oral	Pulp	1	8m	-1.52	+14.33	-31.93	ns	ns	ns	Ohtani et al. (2002)
10	Oral	250	-1	8m	+71.08	+26.03	+140.00	ns	ns	ns	Azuma et al. (1998)
10	Oral	250	0	8m	+354.87	+206.39	+140.00	↑ *	↑ *	ns	Azuma et al. (1998)
10	Oral	250	1	8m	+376.68	+279.45	+20.00	↑ *	↑ *	ns	Azuma et al. (1998)
10	Oral	200	8 hourly (-168 - 0)	8 (5m)	+311.01	+391.67	+12.67	↑ **	↑ ***	ns	Takanaga et al. (2000)
10	Oral	200	9 hourly (-168 - 0), 14	9 (5m)	+131.75	+195.83	-33.33	↑ **	↑ ***	↓ **	Takanaga et al. (2000)
10	Oral	200	10 hourly (-168 - 0), 38	10 (5m)	+69.01	+87.50	+0.00	↑ **	↑ ***	ns	Takanaga et al. (2000)
10	Oral	200	11 hourly (-168 - 0), 72	11 (5m)	+37.00	+25.00	-41.67	ns	ns	↓ **	Takanaga et al. (2000)
10	Oral	200	12 hourly (-168 - 0), 96	12 (5m)	+38.28	+66.67	-14.00	ns	↑ *	ns	Takanaga et al. (2000)
20 SR	Oral	250	0	12	+98.00	+306.00	-53.00				Bailey et al. (1993)

Dose of Drug (mg)	Route	Volume of GJ (ml)	Time of GJ (hr)	Study Group	Mean change as % control AUC	Cmax	tmax	Significance of change AUC	Cmax	tmax	Reference
Nitrendipine											
10	Oral	200	0	9	+38.46	+40.00	+77.00	↑ **	ns	ns	Bailey et al. (1992)
20	Oral	150	-15, -10, -0.25, 5, 10	9	+125.47	+106.25	+26.67	↑ **	↑ **	ns	Soons et al. (1991)
Phenytoin											
300	Oral	300	0	10	NO	NO	NO				Kumar et al. (1999)
Pranidipine											
2	Oral	250	0	16m	YES	YES	NO				Hashimoto et al. (1998)
Pravastatin											
40	Oral	200	8 hourly (-48-0), 0.5, 1.5, 24, 32, 48, 56, 64, 72	11	-7.97	-5.96	+100.00	ns	ns	ns	Lilja et al. (1999) & Neuvonen et al. (1999)
Praziquantel											
180	Oral	250	0	18	+90.19	+62.72	+18.64	↑ *	↑ *	ns	Castro et al. (2002)
Prednisone											
10		150	3 hourly (-7.5-+22.5)	12 (8m) RTP	+50.00	+38.78	-28.63	ns	ns	ns	Hollander et al. (1995)
Quinidine											
200	Oral	250	12 hourly (-48-+96)	6m	NA	+5.00	+26.67		ns	ns	Damkier et al. (1999)
400	Oral	232	0	12	+8.42	-6.67	+106.25	ns	ns	↑ ***	Min et al. (1996b)
Quinine sulfate											
600	Oral	100	12 hourly (-120-0)	10	-23.08	-16.13	-4.35	ns	ns	ns	Ho et al. (1999)
600	Oral	200	12 hourly (-120-0)	10	-3.85	-3.23	+8.69	ns	ns	ns	Ho et al. (1999)
Saquinavir											
600	Oral	150	0, 1	12	+39.00	+63.00	NA				Kupferschmidt et al. (1998)
600	Oral	300	0, 1	12	+121.00	+120.00	NA				Kupferschmidt et al. (1998)

Dose of Drug (mg)	Route	Volume of GJ (ml)	Time of GJ (hr)	Study Group	Mean change as % control AUC	Cmax	tmax	Significance of change AUC	Cmax	tmax	Reference
Simvastatin											
40	Oral	200	0	10	+1,241.72	+1,104.30	+100.00	↑ ***	↑ ***	↑ *	Lilja et al. (2000b)
40	Oral	200	-24	10	+102.16	+136.56	-25.00	↑ ***	↑ **	ns	Lilja et al. (2000b)
40	Oral	200	-72	10	+35.25	+52.69	-25.00	ns	ns	ns	Lilja et al. (2000b)
40	Oral	200	-168	10	+7.91	+33.33	-25.00	ns	ns	ns	Lilja et al. (2000b)
40	Oral	200	8 hourly (-48-0), 0.5, 1.5	10-12	+0.00			ns			Neuvonen et al. (1999)
60	Oral	400	8 hourly (-48-0), 0.5, 1.5	10	+1,513.73	+842.31	+150.00	↑ *	↑ **	ns	Lilja et al. (1998a)
Terfenadine											
60	Oral	250	0	12,	NA	+307.14	NA		↑ **		Rau et al. (1997)
60	Oral	500	0	12,	NA	+350.00	NA		↑ *		Rau et al. (1997)
60 bid 1w	Oral	480	12 hourly (-120 - 0)	6 (3m)	+27.82	+17.39	NA	↑ *	ns		Honig et al. (1996)
60 bid 2w	Oral	480	12 hourly (-144 - 0)	6	+ ¥	NA	NA	↑ ∞			Benton et al. (1996)
60 bid 2w	Oral	480	12 hourly (-142 - +2)	6	+ ¥	NA	NA	↑ ∞			Benton et al. (1996)
120	Oral	300	-0.5	6	+149.08	+242.86	+301.14	↑ *	↑ *	↑ *	Clifford et al. (1997)
Theophylline											
200	Oral	100	0, 1	12	+3.50	-9.68	+0.00	ns	ns	ns	Fuhr et al. (1995)
300 SR	Oral	300	0		Reduced	Reduced	Increased				Gupta et al. (1999)
Triazolam											
0.25	Oral	250	0	10 (6m),	+48.35	+25.00	+67.00	↑ ***	↑ *	↑ *	Hukkinen et al. (1995)
0.25	Oral	200	0	12	+49.47	+37.50	+33.33	↑ **	↑ **	ns	Lilja et al. (2000 A)
0.25	Oral	400	0	12	+45.26	+25.00	+33.33	↑ **	↑ *	ns	Lilja et al. (2000 A)
0.25	Oral	400	8 hourly (-72 - 0)	12	+121.05	+37.50	+33.33	↑ ***	↑ **	ns	Lilja et al. (2000 A)
Verapamil											
60-120	Oral	200	-1	10	+17.91	-15.00	+63.89	ns	ns	↑ *	Zaidenstein et al. (1998)
120	Oral	250	-48, -45, -40, -36, -24, -21, -16, -12, 0, 3, 8, 12	24 (12m)	+42.00	+61.00	+0.00				Fuhr et al. (1994)
120 S	Oral	200	12 hourly (-120 - 0)	9m	+35.81	+57.69	-17.82	↑ *	ns	ns	Ho et al. (2000)
120 R	Oral	200	12 hourly (-120 - 0)	9m	+26.75	+40.43	+7.50	↑ *	ns	ns	Ho et al. (2000)

APPENDIX 3. CALCULATION OF IC_{50}

A3.1 Experimental design

Each experiment was repeated three or four times and a mean and standard deviation were calculated.

A3.2 Radioactivity in cells

The radioactivity in each vial was used to calculate the total radioactivity in each well. If the radioactivity in the C vial is larger than the radioactivity in the W2 vial, it can be concluded that the radioactivity in vial C came from inside the cells and was not just left on the surface of the cells.

As the total amount of radioactivity that the well received was different for each well, the radioactivity in the cells for each well was corrected to the value it would have been if all the wells had received the same total radioactivity.

A3.3 Amount of Gly-Pro in cells

Once the corrected radioactivity in the cells for each well was calculated, the radioactivity was then turned into an amount of Gly-Pro, using Equation A3.1 derived below.

Equation A3.1 Calculation of amount of Gly-Pro for radioactivity

1 dpm = 4.545×10^{-13} Ci

1 pmol = 1×10^{-12} mol

The specific activity of [^{3}H]-Gly-Pro was 48.5 Ci / mmole.

48.5 Ci = 1×10^{-3} mol

$\therefore$ 1 Ci = $(1/48.5 = 0.021) \times 10^{-3}$ mol

$\therefore$ 1 dpm = 4.545×10^{-13} Ci = $4.545 \times 10^{-13} \times 0.021 \times 10^{-3}$ mol = 9.371×10^{-18} mol

$\therefore$ 1 dpm = 9.371×10^{-6} pmol

i.e. pmol = dpm * 9.371×10^{-6}

A3.4 Percentage control activity and percentage control inhibition

Equation A3.2 and Equation A3.3 below were used to calculate the percentage control activity and percentage inhibition for each well. The mean amount of Gly-Pro in the four wells with no inhibitor was calculated and used in Equation A3.2.

Equation A3.2 Calculation of percentage of control activity

$$\%Control = \frac{Amount\_in\_cells}{Amount\_in\_control\_cells} * 100\%$$

Where,

$\%Control$ = Percentage control activity for each well (%)

$Amount\_in\_cells$ = Corrected amount in well (pmol)

$Amount\_in\_control\_cells$ = Mean of corrected amounts in control wells (pmol)

Equation A3.3 Calculation of percentage inhibition of control activity

$$\%Inhibition = 100 - \%Control$$

where,

$\%Inhibition$ = Percentage inhibition of control activity for well

$\%Control$ = Percentage of control activity for well (from Equation A1.3)

A3.5 Calculation of IC_{50}

This value is the concentration of an inhibitor that will reduce uptake by 50%. The value was calculated using Equation A3.4 below. The inhibitor concentration tested was 1mM.

Equation A3.4 Calculation of IC_{50}

$$IC_{50} = \frac{([I])}{\left(\left(\frac{Amount\_in\_control\_cells}{Amount\_in\_cells}\right) - 1\right)}$$

where,

IC_{50} = Inhibitory concentration (50%) (mM)

$[I]$ = Concentration of inhibitor (mM)

$Amount\_in\_cells$ = Corrected amount in well (pmol)

$Amount\_in\_control\_cells$ = Mean of corrected amounts in control wells (pmol)

APPENDIX 4. STATISTICAL ANALYSIS OF CORRELATION BETWEEN MOLECULAR PARAMETERS

A4.1 Key to Analysis

The abbreviations used in the following table are explained below.

IC50	The calculated concentration to inhibit 50% of uptake	Derived in Chapter 3
MWM MW1 MW2 MW3	Molecular Weight of the molecule (M) R_1 group (1), R_2 group (2) and R_3 group (3)	The sum of the atomic weights of all the constituent atoms
LOGPM LOGP1 LOGP2 LOGP3	cLogP of the molecule (M) R_1 group (1), R_2 group (2) and R_3 group (3)	The Log octanol/water coefficient – a measure of hydrophobicity
MRM MR1 MR2 MR3	Molar Refractivity of the molecule (M) R_1 group (1), R_2 group (2) and R_3 group (3)	Related to the molecular size and polarity
MSM MS1 MS2 MS3	Molecular Surface Area of the molecule (M) R_1 group (1), R_2 group (2) and R_3 group (3)	The surface area of the molecule
DIPM DIP1 DIP2 DIP3	Dipole moment of the molecule (M) R_1 group (1), R_2 group (2) and R_3 group (3)	The polarity of the molecule – calculated using CACHe
CDIP	Calculated dipole	The polarity of the molecule – calculated using TSAR
STE	Steric energy	The sum of the energies involved in bond stretching, bond angle bending, and van der Waals and electrostastic interactions
HOMO	Highest Occupied Molecular Orbit	Rlated to electron donation
LUMO	Lowest Unoccupied Molecular Orbit	Rlated to electron accepting
HOF	Heat of Formation	The theoretical energy needed to create the molecule from elements
DON	Donor	The number of hydrogen atoms attached to N, O or S
ACC	Accept	The number of H-bond acceptor groups (N, O, S, F, Cl, Br, I)

Significant correlations are highlighted in either pale blue ($p<0.05$) or dark blue ($p<0.01$).

		IC50	MWM	MW1	MW2	MW3	LOGPM	LOGP1	LOGP2	LOGP3	MRM	MR1	MR2	MR3	MSM	MS1	MS2	MS3	DIPM	DIP1	DIP2	DIP3	CDIP
MWM	Pearson	0.062																					
	Sig.	0.625																					
	N	64																					
MW1	Pearson	0.002	0.734																				
	Sig.	0.990	0.000																				
	N	64	64																				
MW2	Pearson	0.311	0.334	0.176																			
	Sig.	0.012	0.007	0.164																			
	N	64	64	64																			
MW3	Pearson	-0.072	0.690	0.147	-0.177																		
	Sig.	0.574	0.000	0.246	0.162																		
	N	64	64	64	64																		
LOGPM	Pearson	-0.020	0.883	0.548	0.046	0.827																	
	Sig.	0.875	0.000	0.000	0.717	0.000																	
	N	64	64	64	64	64																	
LOGP1	Pearson	-0.045	0.518	0.747	-0.035	0.153	0.641																
	Sig.	0.723	0.000	0.000	0.786	0.228	0.000																
	N	64	64	64	64	64	64																
LOGP2	Pearson	0.311	0.334	0.176	1.000	-0.177	0.046	-0.035															
	Sig.	0.012	0.007	0.164	0.000	0.162	0.717	0.786															
	N	64	64	64	64	64	64	64															
LOGP3	Pearson	-0.084	0.679	0.148	-0.203	0.996	0.827	0.157	-0.203														
	Sig.	0.511	0.000	0.244	0.108	0.000	0.000	0.214	0.108														
	N	64	64	64	64	64	64	64	64														
MRM	Pearson	0.027	0.994	0.701	0.291	0.732	0.915	0.534	0.291	0.720													
	Sig.	0.834	0.000	0.000	0.020	0.000	0.000	0.000	0.020	0.000													
	N	64	64	64	64	64	64	64	64	64													
MR1	Pearson	-0.052	0.708	0.985	0.138	0.140	0.564	0.803	0.138	0.142	0.692												
	Sig.	0.684	0.000	0.000	0.275	0.268	0.000	0.000	0.275	0.263	0.000												
	N	64	64	64	64	64	64	64	64	64	64												
MR2	Pearson	0.311	0.334	0.176	1.000	-0.177	0.046	-0.035	1.000	-0.203	0.291	0.138											
	Sig.	0.012	0.007	0.164	.	0.162	0.717	0.786	0.000	0.108	0.020	0.275											
	N	64	64	64	64	64	64	64	64	64	64	64											

		IC50	MWM	MW1	MW2	MW3	LOGPM	LOGP1	LOGP2	LOGP3	MRM	MR1	MR2	MR3	MSM	MS1	MS2	MS3	DIPM	DIP1	DIP2	DIP3	CDIP
MR3	Pearson	-0.071	0.691	0.147	-0.175	1.000	0.827	0.152	-0.175	0.995	0.732	0.140	-0.175										
	Sig.	0.577	0.000	0.247	0.167	0.000	0.000	0.231	0.167	0.000	0.000	0.270	0.167										
	N	64	64	64	64	64	64	64	64	64	64	64	64										
MSM	Pearson	0.036	0.986	0.733	0.289	0.710	0.918	0.558	0.289	0.694	0.991	0.724	0.289	0.711									
	Sig.	0.784	0.000	0.000	0.026	0.000	0.000	0.000	0.026	0.000	0.000	0.000	0.026	0.000									
	N	59	59	59	59	59	59	59	59	59	59	59	59	59									
MS1	Pearson	-0.003	0.716	0.984	0.123	0.162	0.590	0.825	0.123	0.165	0.695	0.987	0.123	0.161	0.728								
	Sig.	0.980	0.000	0.000	0.335	0.201	0.000	0.000	0.335	0.194	0.000	0.000	0.335	0.203	0.000								
	N	64	64	64	64	64	64	64	64	64	64	64	64	64	59								
MS2	Pearson	0.308	0.335	0.177	1.000	-0.177	0.047	-0.034	1.000	-0.203	0.291	0.139	1.000	-0.175	0.289	0.123							
	Sig.	0.013	0.007	0.162	0.000	0.162	0.714	0.792	0.000	0.108	0.019	0.272	0.000	0.167	0.026	0.331							
	N	64	64	64	64	64	64	64	64	64	64	64	64	64	59	64							
MS3	Pearson	-0.077	0.688	0.141	-0.168	0.997	0.819	0.145	-0.168	0.988	0.730	0.135	-0.168	0.998	0.711	0.154	-0.168						
	Sig.	0.547	0.000	0.267	0.185	0.000	0.000	0.254	0.185	0.000	0.000	0.289	0.185	0.000	0.000	0.223	0.185						
	N	64	64	64	64	64	64	64	64	64	64	64	64	64	59	64	64						
DIPM	Pearson	-0.199	0.119	0.057	0.141	0.053	0.117	0.100	0.141	0.040	0.127	0.072	0.141	0.055	0.197	0.075	0.141	0.065					
	Sig.	0.124	0.363	0.660	0.279	0.683	0.369	0.442	0.279	0.758	0.331	0.583	0.279	0.674	0.146	0.565	0.279	0.617					
	N	61	61	61	61	61	61	61	61	61	61	61	61	61	56	61	61	61					
DIP1	Pearson	0.209	0.382	0.580	0.187	-0.026	0.178	0.297	0.187	-0.027	0.310	0.489	0.187	-0.026	0.308	0.553	0.187	-0.024	0.137				
	Sig.	0.105	0.002	0.000	0.149	0.840	0.170	0.020	0.149	0.834	0.015	0.000	0.149	0.840	0.021	0.000	0.148	0.852	0.294				
	N	61	61	61	61	61	61	61	61	61	61	61	61	61	56	61	61	61	61				
DIP2	Pearson	0.313	0.359	0.195	1.000	-0.174	0.066	-0.020	1.000	-0.202	0.317	0.160	1.000	-0.172	0.318	0.139	1.000	-0.164	0.141	0.187			
	Sig.	0.014	0.005	0.133	.	0.179	0.615	0.880	.	0.118	0.013	0.217	.	0.186	0.017	0.284	0.000	0.207	0.279	0.149			
	N	61	61	61	61	61	61	61	61	61	61	61	61	61	56	61	61	61	61	61			
DIP3	Pearson	0.035	0.340	0.205	-0.120	0.408	0.419	0.204	-0.120	0.434	0.347	0.196	-0.120	0.404	0.367	0.210	-0.121	0.360	-0.062	0.023	-0.120		
	Sig.	0.790	0.007	0.114	0.356	0.001	0.001	0.115	0.356	0.000	0.006	0.130	0.356	0.001	0.005	0.105	0.355	0.004	0.635	0.858	0.356		
	N	61	61	61	61	61	61	61	61	61	61	61	61	61	56	61	61	61	61	61	61		
CDIP	Pearson	0.031	0.138	0.103	0.311	-0.046	0.054	0.027	0.311	-0.038	0.119	0.087	0.311	-0.047	0.134	0.119	0.310	-0.056	0.614	0.175	0.307	0.129	
	Sig.	0.808	0.278	0.417	0.012	0.717	0.674	0.834	0.012	0.764	0.348	0.496	0.012	0.710	0.311	0.347	0.013	0.660	0.000	0.177	0.016	0.322	
	N	64	64	64	64	64	64	64	64	64	64	64	64	64	59	64	64	64	61	61	61	61	
CLOGP	Pearson	-0.020	0.883	0.548	0.047	0.827	1.000	0.641	0.047	0.827	0.915	0.564	0.047	0.827	0.918	0.590	0.047	0.819	0.117	0.178	0.066	0.419	0.054
	Sig.	0.874	0.000	0.000	0.715	0.000	0.000	0.000	0.715	0.000	0.000	0.000	0.715	0.000	0.000	0.000	0.712	0.000	0.368	0.170	0.613	0.001	0.672
	N	64	64	64	64	64	64	64	64	64	64	64	64	64	59	64	64	64	61	61	61	61	64

		IC50	MWM	MW1	MW2	MW3	LOGPM	LOGP1	LOGP2	LOGP3	MRM	MR1	MR2	MR3	MSM	MS1	MS2	MS3	DIPM	DIP1	DIP2	DIP3	CDIP
CMW	Pearson	0.062	1.000	0.734	0.334	0.690	0.883	0.518	0.334	0.679	0.994	0.708	0.334	0.691	0.986	0.716	0.335	0.688	0.119	0.382	0.359	0.340	0.138
	Sig.	0.625	0.000	0.000	0.007	0.000	0.000	0.000	0.007	0.000	0.000	0.000	0.007	0.000	0.000	0.000	0.007	0.000	0.363	0.002	0.005	0.007	0.278
	N	64	64	64	64	64	64	64	64	64	64	64	64	64	59	64	64	64	61	61	61	61	64
STE	Pearson	-0.163	0.720	0.400	0.270	0.592	0.638	0.231	0.270	0.577	0.754	0.437	0.270	0.593	0.756	0.356	0.270	0.597	0.045	-0.119	0.291	0.312	-0.088
	Sig.	0.198	0.000	0.001	0.031	0.000	0.000	0.066	0.031	0.000	0.000	0.000	0.031	0.000	0.000	0.004	0.031	0.000	0.728	0.363	0.023	0.014	0.489
	N	64	64	64	64	64	64	64	64	64	64	64	64	64	59	64	64	64	61	61	61	61	64
HOMO	Pearson	-0.175	-0.040	-0.073	-0.026	0.017	-0.043	-0.090	-0.026	0.012	0.017	0.023	-0.026	0.018	0.007	-0.057	-0.026	0.027	0.102	-0.340	-0.014	-0.203	-0.031
	Sig.	0.166	0.756	0.566	0.839	0.893	0.734	0.479	0.839	0.927	0.896	0.855	0.839	0.889	0.959	0.652	0.837	0.830	0.432	0.007	0.913	0.117	0.811
	N	64	64	64	64	64	64	64	64	64	64	64	64	64	59	64	64	64	61	61	61	61	64
LUMO	Pearson	0.048	-0.035	-0.153	-0.107	0.135	0.106	0.050	-0.107	0.132	-0.038	-0.181	-0.107	0.135	-0.022	-0.086	-0.107	0.133	0.076	-0.036	-0.146	0.121	-0.001
	Sig.	0.708	0.785	0.228	0.402	0.286	0.403	0.696	0.402	0.299	0.765	0.152	0.402	0.286	0.867	0.502	0.402	0.294	0.560	0.781	0.262	0.351	0.996
	N	64	64	64	64	64	64	64	64	64	64	64	64	64	59	64	64	64	61	61	61	61	64
CMR	Pearson	0.027	0.994	0.701	0.291	0.732	0.915	0.534	0.291	0.720	1.000	0.692	0.291	0.732	0.991	0.695	0.291	0.730	0.127	0.310	0.317	0.347	0.119
	Sig.	0.834	0.000	0.000	0.020	0.000	0.000	0.000	0.020	0.000	0.000	0.000	0.020	0.000	0.000	0.000	0.020	0.000	0.331	0.015	0.013	0.006	0.348
	N	64	64	64	64	64	64	64	64	64	64	64	64	64	59	64	64	64	61	61	61	61	64
HOF	Pearson	-0.269	-0.722	-0.656	-0.377	-0.319	-0.536	-0.384	-0.377	-0.316	-0.651	-0.541	-0.377	-0.318	-0.663	-0.622	-0.377	-0.310	-0.088	-0.712	-0.385	-0.247	-0.241
	Sig.	0.032	0.000	0.000	0.002	0.010	0.000	0.002	0.002	0.011	0.000	0.000	0.002	0.010	0.000	0.000	0.002	0.013	0.499	0.000	0.002	0.055	0.055
	N	64	64	64	64	64	64	64	64	64	64	64	64	64	59	64	64	64	61	61	61	61	64
DON	Pearson	-0.149	-0.447	-0.239	0.014	-0.471	-0.621	-0.538	0.014	-0.432	-0.477	-0.262	0.014	-0.474	-0.491	-0.327	0.014	-0.484	-0.173	-0.134	0.002	-0.078	-0.030
	Sig.	0.240	0.000	0.057	0.910	0.000	0.000	0.000	0.910	0.000	0.000	0.037	0.910	0.000	0.000	0.008	0.913	0.000	0.181	0.304	0.985	0.549	0.814
	N	64	64	64	64	64	64	64	64	64	64	64	64	64	59	64	64	64	61	61	61	61	64
ACC	Pearson	0.248	0.519	0.575	0.520	0.009	0.162	0.088	0.520	-0.002	0.428	0.447	0.520	0.010	0.450	0.486	0.520	0.009	0.028	0.630	0.521	0.114	0.211
	Sig.	0.048	0.000	0.000	0.000	0.942	0.202	0.487	0.000	0.988	0.000	0.000	0.000	0.937	0.000	0.000	0.000	0.946	0.832	0.000	0.000	0.381	0.095
	N	64	64	64	64	64	64	64	64	64	64	64	64	64	59	64	64	64	61	61	61	61	64

		CLOGP	CMW	STE	HOMO	LUMO	CMR	HOF	DON
CMW	Pearson	0.883							
	Sig.	0.000							
	N	64							
STE	Pearson	0.638	0.720						
	Sig.	0.000	0.000						
	N	64	64						
HOMO	Pearson	-0.043	-0.040	0.223					
	Sig.	0.735	0.756	0.077					
	N	64	64	64					
LUMO	Pearson	0.106	-0.035	-0.189	-0.547				
	Sig.	0.404	0.785	0.135	0.000				
	N	64	64	64	64				
CMR	Pearson	0.915	0.994	0.754	0.017	-0.038			
	Sig.	0.000	0.000	0.000	0.896	0.765			
	N	64	64	64	64	64			
HOF	Pearson	-0.536	-0.722	-0.174	0.513	-0.276	-0.651		
	Sig.	0.000	0.000	0.170	0.000	0.027	0.000		
	N	64	64	64	64	64	64		
DON	Pearson	-0.621	-0.447	-0.068	0.012	-0.262	-0.477	0.313	
	Sig.	0.000	0.000	0.592	0.922	0.036	0.000	0.012	
	N	64	64	64	64	64	64	64	
ACC	Pearson	0.162	0.520	0.168	-0.499	0.199	0.428	-0.855	0.084
	Sig.	0.202	0.000	0.184	0.000	0.115	0.000	0.000	0.510
	N	64	64	64	64	64	64	64	64