DELIVERY AND STABILITY OF ANTISENSE OLIGONUCLEOTIDE CONJUGATES *IN VITRO*

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SUMMARY

The efficacy of antisense oligonucleotide (ODN) therapy is dependent on four major parameters: delivery to cells, intracellular stability and localisation and efficient action at the target site. The aim of this project was to study the delivery of ODNs to macrophages and to assess the stability of two ODN conjugates, *in vitro*. The first conjugate aimed to improve uptake of ODNs via mannose receptor mediated delivery, the second investigated the improved delivery of ODN conjugates via non-specific lipophilic interaction with the cell membrane. A mono-mannose phosphoramidite derivative was designed and synthesised and a mono-mannose ODN conjugate synthesised by standard phosphoramidite chemistry. This conjugation procedure eliminated the use of bulky protein components often used for mannose conjugation to ODNs. Delivery of this conjugate was enhanced to RAW264.7 and J774 macrophage cell lines via a mechanism of receptor mediated endocytosis. The delivery of three lipophilic ODN conjugates, cholesterol (cholhex), 16-carbon alkyl chain (C_{16}) and hexa-ethylene glycol (HEG) moieties and an unconjugated ODN were assessed in RAW264.7 macrophages. All three conjugates increased the lipophilicity of the ODN as assessed from partition coefficient data. Both the cholhex and unconjugated ODNs were found to have higher degrees of cellular association than the C_{16} and HEG conjugates. Cellular uptake studies implicated internalisation of these ODNs by an adsorptive endocytosis mechanism. Three putative ODN-binding proteins were identified in RAW264.7 cell membranes. Following endocytosis, ODNs must remain stable during their residence in endosomal/lysosomal compartments prior to exiting and exerting their biological action in either the cytosol or nucleus. Assessment of *in vitro* stability in a lysosomal extract revealed the cholhex conjugate and unconjugated ODNs to have a longer half-life than the C_{16} and HEG conjugated ODNs, highlighting the influence of conjugate moieties on lysosomal stability. The effects of base composition and length on stability in a lysosomal extract revealed the longest half-life for homo-cytidine ODNs and ODNs over 20 nucleotides in length. Cellular association studies revealed homo-ODNs to bind in the order G<T<C<A, a plateauing of binding observed for ODNs over 20 nucleotides in length. These studies suggest that the above conjugates can enhance cellular association and delivery of antisense ODNs to cultured macrophages. This may lead to their use in treating disorders such as HIV infection, which affects this cell type.

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ABBREVIATIONS

A, C, G, T	adenine, cytosine, guanine, thymidine
A_{580}	absorbance 580 nm
aa	amino acid
AMPS	ammonium persulphate
ATP	adenosine-5'-triphosphate
bFGF	basic fibroblast growth factor
bp	base pairs
BSA	bovine serum albumin
cDNA	complementary DNA
Ci, mCi, μCi	Curie, milliCurie, microCurie
Cm, mm, μm, nm	centimetre, millimetre, micrometre, nanometre
dATP	deoxyadenosine triphosphate
2DG	2-deoxy glucose
ddATP	dideoxyadenosine triphosphate
DNA	deoxyribonucleic acid
DMSO	dimethylsulphoxide
DMT	dimethoxytrityl
DNase	deoxyribonuclease
EDTA	ethylenediaminetetra-acetic acid
FCS	foetal calf serum
g, mg, μg, ng	gram, milligram, microgram, nanogram
HBSS	Hanks buffered saline solution
HIV	Human immunodeficiency virus
hr	hour
im	intramuscular

ip	intraperitoneal
iv	intravenous
kDa	kiloDaltons
L, ml, µl	litre, millilitre, microlitre
M, mM, µM, nM	molar, millimolar, micromolar, nanomolar
mmol, µmol, nmol, pmol	millimoles, micromole, nanomole, picomole
min	minutes
MEM	minimum essential medium-eagle
mRNA	messenger RNA
MP	methylphosphonate
MW	molecular weight
NaN_3	sodium azide
OD	optical density
ODN	oligonucleotide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PEG	polyethylene glycol
PLL	poly-lysine
PO	phosphodiester
PS	phosphorothioate
rpm	revolutions per minute
RNA	ribonucleic acid
RNase	ribonuclease
RSV	Rous sarcoma virus
RT	reverse transcriptase
28S*rev*	28-mer phosphorothioate antisense *rev* oligonucleotide
SDS	sodium dodecyl sulphate

sec	seconds
$t^1/_2$	half life
TBE	Tris-borate EDTA electrophoresis buffer
TEMED	N, N, N', N' -tetramethylethylenediamine
T_m	melting temperature
Tris	Tris (hydroxymethyl) aminomethane
u.v.	Ultraviolet
V	Volts

CHAPTER 1: GENERAL INTRODUCTION

1.1 THE CONCEPT OF ANTISENSE THERAPY

Many clinical disorders and diseases can be attributed to aberrant gene function, be it under- or over-expression, point mutations or gene re-arrangements. A significant number of conditions involve the expression of undesirable genes. Antisense therapy promises the selective inhibition of gene expression at the molecular level, distinguishing itself from other therapies due to the high degree of selectivity available. The rationale of antisense therapy is based on the process of gene expression, the precise hybridisation of the antisense agent with a complementary target, interfering with protein production.

1.1.1 Gene Expression

The process of gene expression involves three main stages which can all potentially be inhibited by antisense agents. A schematic representation of the events is shown in figure 1.1. The first stage is transcription of a gene into pre-messenger RNA (pre-mRNA), this transcript codes for both exons (coding regions) and introns (non-coding regions). The pre-mRNA molecule then undergoes RNA processing whereby intron regions are removed and the exon regions ligated to form mature messenger RNA (mRNA). Both transcription and RNA processing are complex events that occur in the nucleus. The final stage, translation, involves synthesis of a polypeptide from the mRNA. The amino acid sequence of the polypeptide is encoded by the nucleotide sequence of the mRNA, there being four different nucleotides, guanosine (G), cytidine (C), thymidine (T) and adenosine (A).

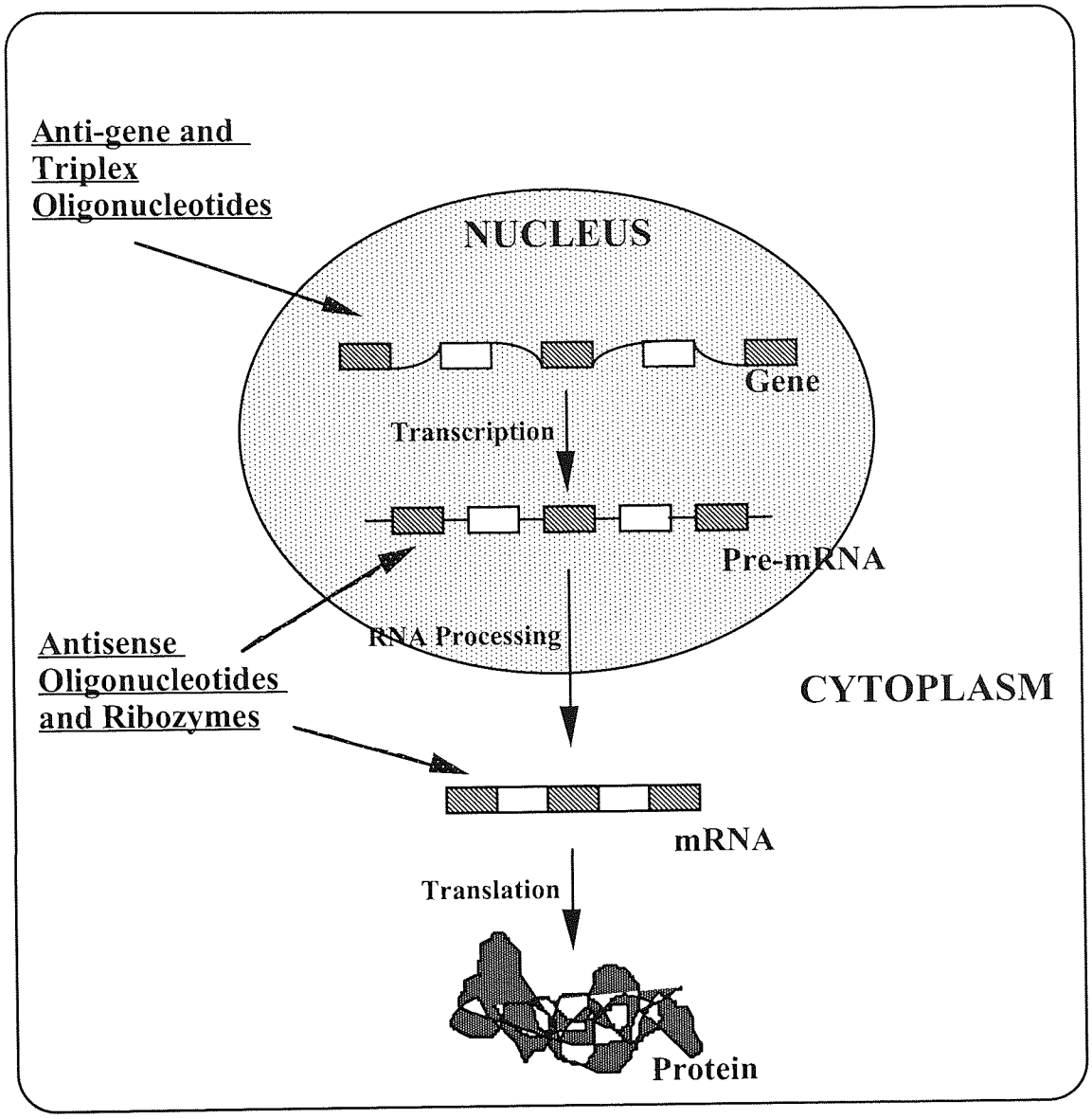


Figure 1.1: Schematic diagram of Gene Expression. A gene is transcribed into pre-mRNA, which is subsequently processed to remove non-coding regions (thin lines between boxes) resulting in mRNA. Finally, the mRNA is translated to produce a protein. The potential sites for antisense action are shown. Open and striped boxes represent coding regions. Diagram adapted from Akhtar *et al.* (1991a).

1.1.2 Hybridisation of Complementary Nucleic Acid Sequences

Hybridisation between complementary nucleic acid sequences occurs by what is known as Watson-Crick base pairing. When in an appropriate orientation the G-nucleotide is able to form three hydrogen bonds with a C-nucleotide, and the T-

nucleotide from two hydrogen bonds with an A-nucleotide (figure 1.2). Due to this phenomenon, G and C, and T and A nucleotides are referred to as complementary to each other. The formation of such hydrogen bonds between nucleic acids is the principle on which antisense therapy is based, aswell as being the fundamental basis for molecular genetics (the expression of biological information by the cell).

1.1.3 The First Antisense Experiments

Oligonucleotides (ODN) are short stretches of synthetic single stranded DNA, designed to be complementary to a specific target. The first experiment whereby gene expression was affected by an antisense ODN was conducted by Barrett *et al.* (1974). An ODN was designed as antisense to transfer RNA (tRNA), hybridisation blocked enzymic recognition and subsequently disrupted aminoacylation in a cell free system. Izant *et al.* (1984) observed the same antisense effect in murine cells, which were microinjected with a plasmid carrying antisense RNA, inhibiting expression of the thymidine kinase gene. A proliferation of antisense experiments involving ODNs followed, Zamecnik *et al.* (1978) inhibited viral replication *in vitro* with an ODN, Kawasaki (1985) co-injected Xenopus oocytes with mRNA and a complementary ODN, leading to inhibition of mRNA expression.

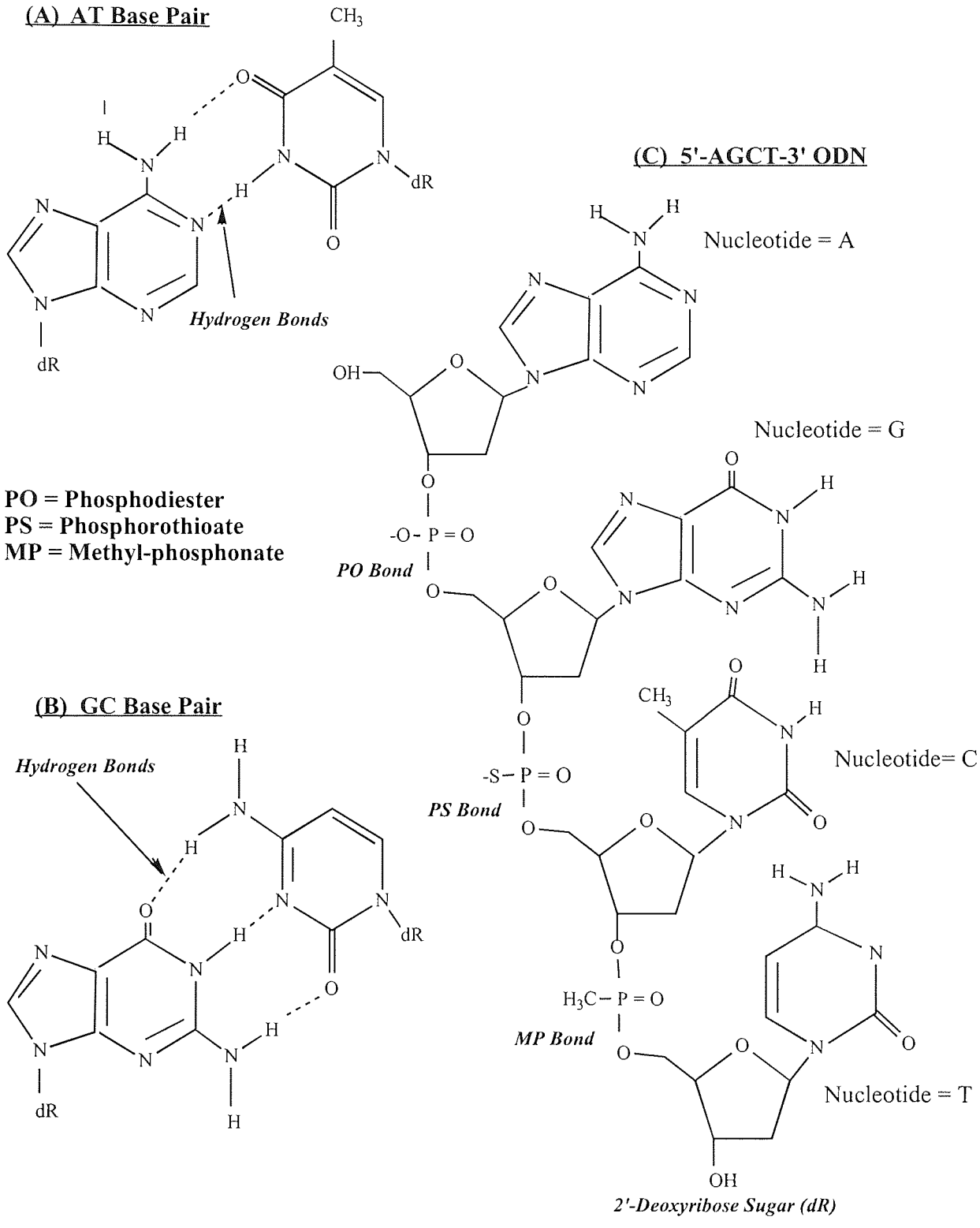


Figure 1.2: Schematic Representation of Watson-Crick Base Pairing and Nucleotide Bases. (A) Hydrogen bonding between AT base pairs, (B) hydrogen bonding between GC base pairs and (C) a short ODN composed of AGCT bases linked by different internucleotide linkages (phosphodiester, PO, phosphorothioate, PS and methylphosphonate, MP respectively). dR = 2'Deoxy-ribose sugar.

1.2 ANTISENSE RNA

Antisense RNA was found as a naturally occurring phenomenon in prokaryotic systems. Lacatena *et al.* (1981) found antisense RNA to modulate DNA replication of ColE1-type plasmid by binding a primer involved in replication, cellular protein levels were decreased by antisense RNA hybridisation to target mRNA causing inhibition of translation. Green *et al.* (1986) reviews these early experiments. Hildebrandt *et al.* (1992) describe an example of post-transcriptional regulation of gene expression where natural antisense RNA functions to regulate mRNA stability. These studies highlight the potential for regulating gene expression by exogenous antisense RNA.

1.3 ANTISENSE AGENTS AND MECHANISMS OF ACTION

As shown in figure 1.1, an antisense agent can target a number of nucleic acid sites along the gene expression pathway. Targetting the gene itself is referred to as the 'anti-gene or triplex strategy'. The 'antisense strategy' refers to the mechanism by which an RNA molecule is targetted. Ribozymes are another class of antisense agent, which act by a unique mechanism to inhibit gene expression.

1.3.1 The Anti-gene Strategy

An antisense agent can be designed as complementary to, and bind duplex DNA by hydrogen bonding. These agents are termed 'triple-helix forming ODNs (TFOs). The result is a structure termed a 'triplex', which has a triple helix configuration (figure 1.2 shows a schematic representation). A triplex forms when hybridisation occurs between a single stranded antisense oligonucleotide and the duplex DNA by Hoogsteen bonding. Hoogsteen bonding refers to the formation of a hydrogen bond (H-bond) between an A-nucleotide of the ODN and A-T base pair of the DNA, a G-nucleotide with G-C base pair of the DNA or a C-nucleotide with C-G of DNA (Cooney *et al.* 1988). Such bonding occurs at the major groove of the duplex, there

being adequate space at this site for the third strand (Sun *et al.* 1989), occurring at purine rich (A- and G-nucleotides) regions of the DNA duplex (Moser *et al.* 1987).

Triplex formation between a TFO and the DNA duplex can result in transcription being blocked, by either preventing initiation or elongation. One of the advantages of the triplex approach is that binding between the TFO and duplex DNA is of a high affinity and therefore the effects are more long term than ODNs binding mRNA. Another advantage to targetting the gene is that there are fewer numbers of potential sites of action in the nucleus compared with the pools of mRNA in the cytoplasm. TFO stability and delivery to the nucleus are two of the major problems encountered with the antigene approach. The TFO must first gain access to the nucleus and subsequently remain intact long enough to bind the target gene.

Moser *et al.* (1987) first demonstrated triplex formation whereby a homopyrimidine ODN was shown to bind a stretch of homopurine duplex DNA. Thereafter, expression of the *c-myc* gene was inhibited by triplex formation *in vitro* by a 27-mer ODN in a study by Cooney *et al.* (1988) and Agrawal *et al.* (1996) inhibited the TNF gene with TFOs and inhibited growth of TNF-dependent tumour cells *in vitro*. Ing *et al.* (1993) revealed that several hundred fold higher concentrations of TFOs were required *in vivo* to acheive the same effects as *in vitro*, a disadvantage of the triplex approach as the chance of non-specific effects is increased with concentration. The potential use of TFOs for cancer therapy is highlighted by these studies.

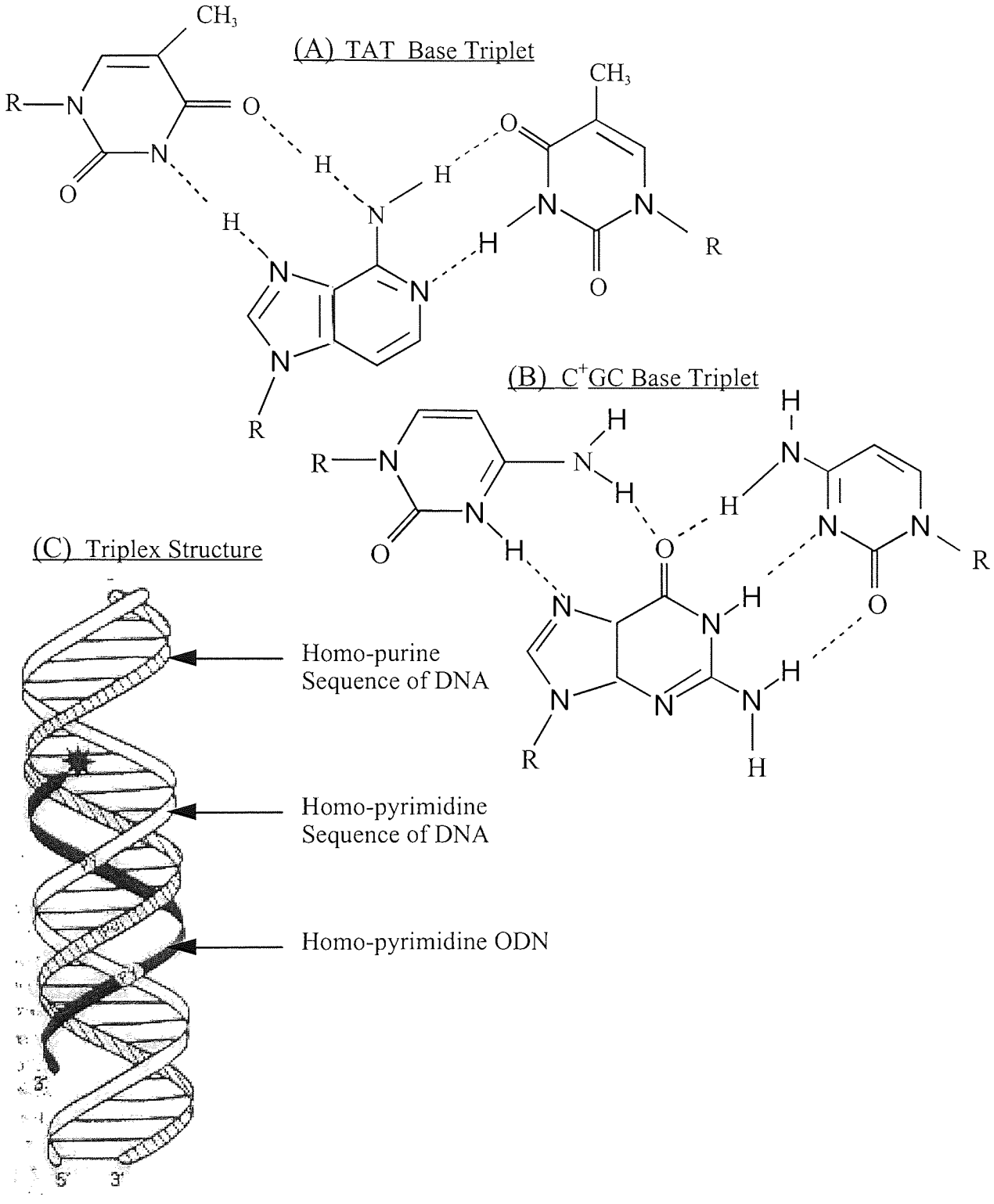


Figure 1.3: Schematic Representation of Hoogsteen Base Pairing and a Triplex Structure. (A) Hoogsteen base paring (left hand side dashed lines) between T and AT bases pairs, (B) Hoogsteen base pairing (left hand side dashed lines) between C and GC base pairs and (C) a homo-pyrimidine ODN in the major groove of double helical DNA of homo-purine/homo-pyrimidine sequence. Diagram 'C' from Helene *et al.* (1990).

1.3.2 The Antisense Strategy

Targetting nuclear pre-mRNA or cytoplasmic mRNA with a complementary ODN is referred to as the 'antisense strategy'. There are two mechanisms by which gene expression can be inhibited. Hybridisation of an antisense ODN to pre-mRNA or mRNA can sterically hinder the next stage of gene expression (Wientraub *et al.* 1990). If pre-mRNA is the target, RNA processing and subsequent maturation to mRNA can be prevented. Ribosomal translation of mRNA can be blocked following antisense ODN hybridisation to mRNA. The other mechanism involves cleavage of the RNA component of the DNA:RNA complex (ODN:mRNA) by the ubiquitous enzyme RNase-H (Bonham *et al.* 1995).

RNase-H plays an important role in DNA replication and translation under normal conditions (Uhlmann *et al.* 1990; Jarosweski *et al.* 1991; Carter *et al.* 1993). RNase-H levels peak during DNA replication and translation, one of its functions is to degrade RNA primers from which DNA replication is initiated. Dash *et al.* (1987) showed RNase-H mediated cleavage of target mRNA resulting in translation arrest in Xenopus oocytes. Woolfe *et al.* (1990) observed RNase-H activity in Xenopus oocytes injected with ODNs, the target mRNA degraded by RNase-H action following hybridisation. Also, Walder *et al.* (1988) showed mRNA degradation by RNase-H at the site of hybridisation with a complementary ODN. Therefore, the action of this enzyme appears to play a key role in ODN inhibition of gene expression. It must be noted that RNase-H is only active in replicating cells, implying that quiescent cells may not possess sufficient levels of this enzyme to act on an ODN:mRNA complex (Neckers *et al.* 1992).

1.3.3 The Ribozyme Strategy

A further class of antisense agent are RNA molecules called ribozymes, that constitute the 'ribozyme strategy'. Ribozymes are small, oligoribonucleotides that possess fast acting catalytic activity (Haseloff *et al.* 1988; Herschlag *et al.* 1990). Their structure differs significantly from DNA ODNs, however their application and types of target

are similar. Ribozymes comprise of a catalytic domain and two flanking substrate-binding domains (figure 1.4). The latter domain is the region that allows catalytic activity to be directed to a target RNA sequence, referred to as trans-cleavage (Koizumi *et al.* 1988), designed such that it is complementary to sites flanking the region to be cleaved. Ribozymes are attractive alternatives to ODNs because of their potential to act on multiple target molecules, like conventional catalysts, however, one important drawback is stability. Like all RNA species, they are prone to rapid degradation by intra- and extra-cellular ribonucleases. The chemistry of ribozymes has been modified in order to confer nuclease resistance (Beigelman *et al.* 1995). Desjardins *et al.* (1996) show ribozymes remaining intact in plasma after 48 hours, supporting the potential use of ribozymes therapeutically.

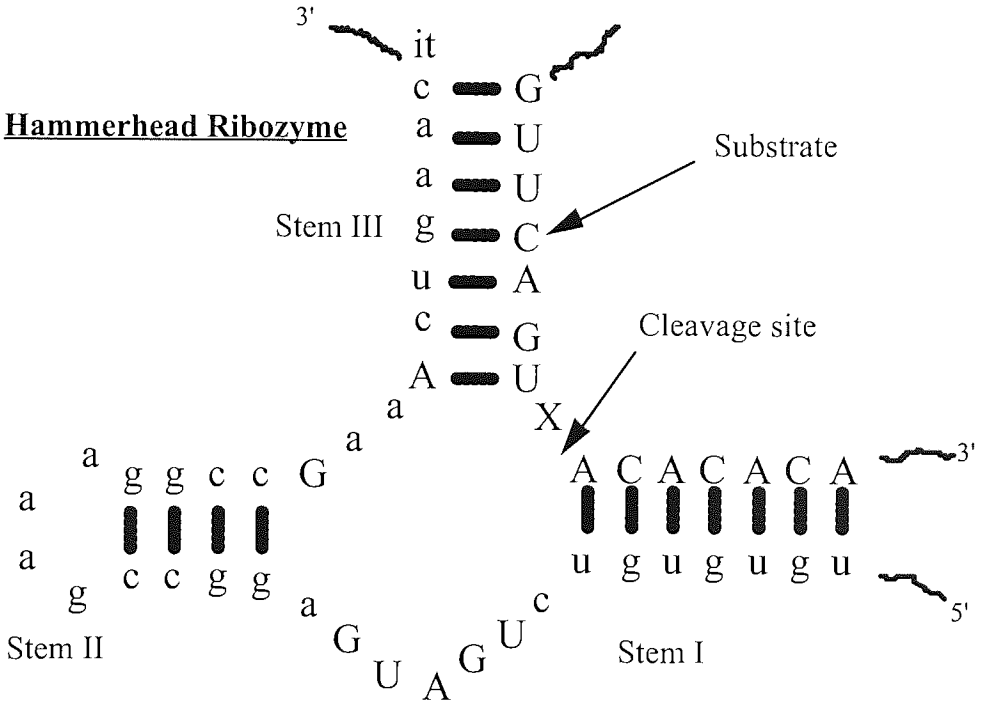


Figure 1.4: The Diagramatic Representation of a Hammerhead Ribozyme. The sequence shows a bound substrate (epidermal growth factor receptor), site of cleavage, antisense base pairing regions (stems I and III, lower case letters) and an internal loop (stem II, lower case letters) responsible for binding divalent cations which is required for catalytic activity. The central core comprises of non-base pairing conserved nucleotides (upper case letters). Cleavage occurs at 3'end of X (A, C or Uridine bases). Diagram courtesy of Ms.P.Fell, Aston University, U.K.

Several classes of ribozyme have been identified, of them the most widely studied are the 'hammerhead' self-cleaving ribozymes of viroids (figure 1.4) and 'hairpin' ribozymes of tabacco ringspot virus, the former found to be effective both *in vitro* and *in vivo* in inhibiting gene expression (reviewed by Marschall *et al.* 1994; Kijima *et al.* 1995; Akhtar *et al.* 1996). Both hammerhead and hairpin ribozymes cleave target mRNA at GUC recognition sites, although other sites can be cleaved.

Catalytic activity and substrate specificity of two hammerhead ribozymes was reported by Scherr *et al.* (1997). These ribozymes targetted the N-ras oncogene, *in vitro* studies revealed that binding and cleavage of the target sequence was highly specific, a single base change abolishing the ability of the ribozyme to cleave the target. A 55% reduction in activity of reporter gene, linked to the N-ras oncogene target, was found *ex-vivo* in cells transfected with a plasmid containing the N-ras/reporter gene construct. These ribozymes were delivered exogenously in a liposomal preparation. Scherr *et al.* (1997) highlights the potential of ribozymes as antisense agents for the specific down-regulation of gene expression.

1.4 TARGET FOR THE ANTISENSE AGENT

The choice of target for an antisense agent is critical in terms of efficacy, for two reasons. Firstly the target site must be such that the antisense molecule will be able to gain easy access and hybridise to it. Nucleic acids, in general, are prone to the formation of secondary/tertiary structures and RNA target sites may naturally adopt such structures *in vivo*. Secondary structures in or adjacent to the target site may sterically hinder hybridisation, rendering the antisense agent ineffective. Computer programs have been designed to predict probable secondary structure formation within a sequence to enable appropriate target sites to be chosen (Han *et al.* 1994). Proteins may also be naturally associated with the target molecule for various reasons, impeding antisense binding, therefore these regions must also be avoided.

Also essential in antisense design is that the sequence of the target region must be exclusive to that gene alone, ensuring that binding of the antisense agent will not occur at non-target sites, thereby eliminating the possibility of non-specific gene down-regulation (Helene *et al.* 1989). Computer programs can also determine the frequency of a particular sequence within a genome, eliminating the possibility of targetting a sequence with an antisense molecule non-specifically (Han *et al.* 1994). An ODN must not be too long otherwise tight binding to mismatched sequences may result, leading to non-specific inhibition of transcription. A number of studies have been performed to evaluate the minimum ODN length required to bind a target sequence exclusively, the ODN therefore only having one target. This length will be determined by the nature of the target, be it pre-mRNA, mRNA or DNA. Predictions are made on the assumption that all four nucleotides, A, C, G and T, are randomly distributed, and also the size of the genome is accounted for. For the human genome, a particular sequence of 17-nucleotides is predicted to occur only once by Helene *et al.* (1989) and Weintrub (1990), other studies state that 11 to 15 bases are optimal depending on base composition. Therefore this length is predicted as an ideal length for an ODN to achieve specific binding to a target site.

1.5 OLIGONUCLEOTIDE STABILITY

Antisense ODNs need to meet a number of criteria if they are to be ultimately used therapeutically *in vivo*. Biological stability, pharmacokinetics and bio-distribution are all important parameters that need to be investigated with respect to any potential new drug. First and foremost the stability of ODNs must be evaluated in the particular system to which they are being applied. Unmodified ODNs, comprising of phosphodiester bonds (termed phosphodiester or PO-ODNs), figure 1.2, are prone to degradation by cellular nuclease enzymes, both extracellularly and intracellularly. The most important type of nuclease enzymes are phosphodiesterases, of which there are two types. Exonucleases cleave the phosphodiester bond, either 5'-end or 3'-end depending on the type, and endonucleases cleave bonds within the molecule often

being sequence specific (for reviews see Uhlmann *et al.* 1990 and Neckers *et al.* 1992).

A great variability exists between different reports in terms of ODN stability. Wickstrom *et al.* (1986) found a PO-ODN 15 bases in length (15-mer) to be stable for up to two hours at 37°C in 5% serum whereas Akhtar *et al.* (1991c) found a 14-mer PO-ODN to degrade within 10 minutes in 10% heat inactivated serum. Akhtar *et al.* (1991c) showed PO-ODN degradation in both cellular cytoplasmic and nuclear extracts. Degradative activity was greatest in the nucleus, therefore suggesting that targetting of an ODN to sites within the nucleus will probably prove less effective than cytoplasmic sites. An *in vivo* assay by Woolfe *et al.* (1990) revealed a PO-ODN, microinjected into Xenopus oocytes, to have a half life of less than 30 minutes, and Fisher *et al.* (1993) found microinjected PO-ODNs to have a similar half life in five different mammalian cell lines.

1.6 OLIGONUCLEOTIDE MODIFICATIONS

Poor stability of PO-ODNs (Wickstrom *et al.* 1986; Woolfe *et al.* 1990; Akhtar *et al.* 1991C) gave rise to the backbone, comprising of phosphodiester bonds, to be modified in order to increase stability to enzymic degradation. One of the non-bridging oxygen groups of the phosphodiester bond can be substituted, thereby prolonging recognition and degradation by nucleases. Modifications to the base, sugar moieties and the conjugation terminal groups have also been performed for the same means. Uhlmann *et al.* (1990), Neckers *et al.* (1992) and Milligan *et al.* (1993) review the multitude of modifications made to ODNs and the nuclease stability conferred.

1.6.1 Phosphorothioate and Phosphorodithioate Oligonucleotides

Of the most studied modifications to the phosphodiester bond is substitution of one of the non-bridging oxygens of the bond with a sulphur, producing a phosphorothioate

(PS ODNs), figure 1.2. PS-ODN synthesis is easily achieved by an automated processes. The negative charge and water solubility of the native PO-ODN is conserved during this modification. However, a number of properties are altered.

Due to the tetrahedral nature of the phosphate atom of the phosphodiester bond, substitution of an oxygen atom results in the formation of two dia-sterioisomers which have different properties with respect to nuclease degradation (Uhlmann *et al.* 1990). The endonuclease, EcoR1, specifically cleaves one dia-sterioisomer in preference to the other, rendering the other resistant to degradation (Gallo *et al.* 1986). Such variation in enzymic stability of diasterioisomers is important when therapeutic efficacy is considered, the exact parameters of a drug must be known if *in vivo* applications are made so as to eliminate batch-batch variation (Milligan *et al.* 1993).

Many studies have shown PS-ODNs to be significantly more stable in biological milieu compared to PO-ODNs (Campbell *et al.* 1990; Shaw *et al.* 1991). Woolfe *et al.* (1990) found a PS-ODN to have a half life 6-fold longer than a PO-ODN counterpart. Studies have shown PS-ODNs to generally have a greater biological stability than PO-ODNs, by factors greater than ten. Stein *et al.* (1988) report the greater ability of PS-ODNs to activate RNase-H compared to PO-ODNs, mRNA degradation being induced by PS-ODN hybridisation and RNase-H action (Woolfe *et al.* 1990). However, the activation of RNase-H does not involve a simple mechanism. Gao *et al.* (1992) report inhibition of RNase-H cleavage at high PS-ODN concentrations, the mechanism be which this ocurrs being unclear but competative inhibition of the enzyme by ODN binding is one theory. Therefore, the target is protected from RNase-H at these high PS-ODN concentrations.

The use of PS-ODNs instead of PO-ODNs entails a number of disadvantages. Hybridisation affinity of PS-ODNs to their targets is reduced (Kibler-Herzog *et al.* 1991), duplexes are less stable than those formed between a PO-ODN and target due to a lower T_m (temperature required to melt apart 50% of the duplex to component strands) (Stein *et al.* 1988, Ghosh *et al.* 1993). Non-specific biological effects are also associated with PS-ODNs due to their greater affinity for binding proteins (Stein *et al.*

1993; Zhao *et al.* 1993; Ehrlich *et al.* 1994). A non-complementary (nonsense) and complementary (antisense) PS-ODN were both found to have an anti-Human immunodeficiency virus (HIV) effect in a study be Matsukura *et al.* (1987). Gao *et al.* (1990 and 1992) showed that PS-ODNs exerted non-sequence specific effects on HIV by binding polymerases. Coulson *et al.* (1996) studied PS-ODN efficacy in epithelial cells where a non-antisense mediated morphological and anti-proliferative change was observed resulting from a sequence-selective effect on tyrosine kinase phosphorylation. Such studies highlight the ability of PS-ODNs to bind proteins, thereby exerting unpredictable affects. It is essential that non-antisense mediated efffects are detected and reduced, this can be acheived by the use of appropriate control sequences and use of pure ODN preparations of optimal base length and composition respectively. PS-ODNs containing four contiguous G-nucleotides have been found to exert non-antisense effects. ODNs with a high G-nucleotide content have the potential to form secondary structures. Peyman *et al.* (1995) report G-quartet loop structures in ODNs containing four contiguous G-nucleotides and Coulson *et al.* (1996) implicate secondary structure formation in a PS-ODN of high G-nucleotide content. Morphological changes and a decrease in growth rate of human epithelial cells incubated with a PS-4G-ODN, accompanied by no corresponding affect on mRNA or protein levels was observed by Yaswen *et al.* (1993). Burgess *et al.* (1995) attributed PS-ODN mediated smooth muscle cell proliferation, *in vitro* and *in vivo*, to the presence of four contiguous G-nucleotides in the ODN sequence, and Coulson *et al.* (1996) attributed the non-antisense effects observed in their study to high G-nucleotide content.

Chimeric ODNs, comprising of a mixture of phosphodiester and phosphorothioate bonds (PS/PO-ODNs) were synthesised to reduce non-specific effects (Stein *et al.* 1988; Hoke *et al.* 1991; Zhao *et al.* 1993; Ehrlich *et al.* 1994). PS/PO-ODNs were found to maintain stability when in a duplex with the target, comparable to full PS-ODNs. However, a reduction in nuclease stability was observed and some antisense activity was lost (Hoke *et al.* 1991). The reasons for diminished antisense activity was attributed to either poor recognition by RNase-H or poor stability to nuclease degradation. Zhao *et al.* (1993) found PS/PO-ODN cellular association to be lower

than the full PS-ODN but higher than full PO-ODNs. A lower degree of non-specific protein binding was observed for a PS/PO-ODN compared to the full PS-ODN counterpart, thus highlighting a major advantage of partial ODN modification (Ehrlich *et al.* 1994). Therefore, it is obvious that the advantages and disadvantages of partial or full PS-ODNs must be balanced in terms of ODN efficacy.

Phosphorodithioates (PS_2-ODNs) have been produced to resolve the problem of isomer formation, whereby both non-bridging oxygen atoms are substituted with sulphur atoms (Ghosh *et al.* 1993; Cummins *et al.* 1996). PS_2-ODNs are chemically stable, have similar nuclease resistance to PS-ODNs (Marshall *et al.* 1993), are able to form duplexes with complementary nucleic acids and able to induce RNase-H action. However, these latter two properties were found to be less efficient than that of PS-ODNs (Cummins *et al.* 1996). Marshall *et al.* (1993) found PS_2-ODNs to inhibit HIV reverse transcriptase by binding to the active site of the enzyme. Although PS_2-ODNs appear to be ideal antisense agents, not all data has proved promising. Ghosh *et al.* (1993) found protein binding of PS_2-ODNs to be of a similar magnitude to PO-ODNs, which was 6-fold lower than the corresponding PS-ODN, PS_2-ODN duplexes were also found to be less stable compared to PS-ODN. Regardless of the conflicting reports of PS-ODN action (antisense or non-antisense mediated) and the possible alternative ODNs, this modification remains be far the most widely used in antisense research.

1.6.2 Methylphosphonate Oligonucleotides

Much work has concentrated on methylphosphonate ODNs (MP-ODNs), produced when a non-bridging oxygen of the phosphodiester bond is substituted with a methylphosphonate group. This modification differs from PS-ODNs in that the ionic nature of the ODN is lost, therefore MP-ODNs are neutral molecules.

Theoretically, based on their neutrality, binding of MP-ODNs to target nucleic acids should be more favorable. There is significantly less repulsion between MP-ODNs

and the phosphate backbone of the target (Neckers *et al.* 1992). However, the methyl groups confer steric hindrance and subsequently reduce ability to form a stable duplex with the target. Compared to PO-ODNs and PS-ODNs, MP-ODNs are poorly water soluble, restricting their therapeutic use (Akhtar *et al.* 1991c). As with PS-ODNs, diasterioisomers are produced during synthesis. Another disadvantage of MP-ODNs is that RNase-H is not activated, therefore higher concentrations are required to confer an antisense affect.

There are a number of advantages to the use of MP-ODNs, they have a non-ionic nature and therefore should be able to gain access to the cell across the membrane more easily than PO-ODNs or PS-ODNs. Shoji *et al.* (1991) showed active uptake of MP-ODNs, the mechanism however differing from that of PO-ODNs and PS-ODNs. MP-ODNs have been found to be very stable to nuclease degradation (Budker *et al.* 1992) and shown to have low toxicity (Shoji *et al.* 1991). Cheng *et al.* (1997) have shown MP-ODNs to bind proteins to a significantly lower affinity than PO-ODNs or PS-ODNs, therefore the probability of non-specific binding is reduced. A number of successful MP-ODN antisense effects have been observed, Blake *et al.* (1985) observed mRNA transaltion inhibition in reticulocyte lysates by an antisense MP-ODN and Agris *et al.* (1986), in a similar cell free system demonstrated the inhibition of Vesicular Stomatis virus by a corresponding antisense MP-ODN. Lin *et al.* (1995) found that modification of an antisense ODN with terminal MP linkages, at the 3'-end and 5'-end, to inhibit alpha-feto protein production in human hepatoma cells by 72% compared to non-chimeric ODNs. These chimeric-MP ODNs were found to be stable in cell culture medium following 4hrs incubation and toxic effects were not observed.

1.6.3 Conjugation of Terminal Groups

As discussed in section 1.5, there exist 5'-end and 3'-end nuclease enzymes which degrade ODNs from their ends. In an attempt to prevent recognition by degradative enzymes, these ends have been modified by the conjugation of specific groups. The

conjugation of terminal groups also serves to enhance cellular ODN uptake in some cases, discussed later in sections 1.9 and 4.1.2.

Thuong *et al.* (1987) conjugated an acridine group to the 5'-end of an ODN, which conferred partial protection from exonuclease degradation while maintaining antisense activity and Saison-Behmoaras *et al.* (1997) conjugated a hydrophobic tail, dodecanol, to the 3'- and 5'-end of a PO-ODN, finding an increase in stability to degradation in rabbit reticulocyte lysate. Goto *et al.* (1997) reported the increased stability to nucleases by ODNs conjugated to either poly-L-lysine or poly-ethylene glycol, *in vitro* and *in vivo*. Naked ODNs were found to degrade within 1 minute compared to 40% of the PLL-conjugated ODN remained intact after 24 hours, when incubated in undiluted human serum. Short and long chained poly-ethylene glycol (PEG) conjugated ODNs were also found to have resistance to nuclease action by Bonara *et al.* (1997). PEG-conjugated 12-mer ODNs were found to have a slower degradation profile in the presence of the hydrolytic enzymes phosphodiesterase and nucleotidase from snake venom, compared to the unconjugated control (Bonara *et al.* 1997). PEG is a non-toxic, soluble molecule which is discussed later in section 4.1.2.2 in terms of enhanceing ODN uptake. A number of ODNs conjugated to different lipophilic groups were found to have a greater resistance to nucleases as reported by Zhang *et al.* (1997).

These are amongst a multitude of studies which demonstrate increased ODN stability, to degradation by nucleases, by the conjugation of terminal groups. Often a dual function of increasing stability alongside cellular uptake can be achieved by such conjugation, and also the effective ODN dose can be reduced.

1.7 OLIGONUCLEOTIDE UPTAKE

Theoretically, the passive diffusion of ODNs across the plasma membrane is not favorable. ODNs are typically 15 to 30 nucleotides (15-mer to 30mer) in length and therefore have high molecular weights (MW) and have a poly-anionic nature, which

makes them unable to permeate the phospholipid bilayer of the membrane (Yakubov *et al.* 1989; Akhtar *et al.* 1992b).

Early ODN uptake experiments were performed without the use of delivery vehicles or strategies to enhance uptake (Zamecnik *et al.* 1978; Wickstrom *et al.* 1988). Successful down-regulation of gene expression was reported in many cases, particularly against viral targets or cellular proteins associated with proliferation. This data must however, be treated with caution when interpreted as a successful antisense experiment. There may not necessarily have been sufficient ODN uptake so as to exert a positive antisense effect. The down-regulation of these viral gene targets may be due to non-specific binding of PS-ODNs to enzymes such as reverse transcriptase, DNA polymerase or viral binding proteins, amongst others. The majority of current work within the antisense field resides in the use of specific delivery systems, table 1.1 lists some studies which show ODN uptake into cells.

Table 1.1: Some Oligonucleotide Uptake Studies

AUTHOR	CELL LINE	ODN
Gao *et al.* (1990)	HeLa	PS
Wu *et al.* (1992)	HepG2 (Human Hepatoma)	PO
Degols *et al.* (1994)	MT4 (Human T-Cell Leukemia)	PO
Beltinger *et al.* (1995)	K562 (Human Leukemia)	PS
Shoji *et al.* (1996)	Vero (Monkey kidney cells)	PO, PS, MP
Lappalainen *et al.* (1997)	CaSki (Human Cervical Cancer)	PO
Rojanasakul *et al.* (1997)	Rat Alveolar Macrophages	PS

1.7.1 Uptake of Unmodified Oligonucleotides

A number of studies report uptake of unmodified ODNs into cells to a sufficient degree to mediate an effect on intracellular mRNA (Zamecnik *et al.* 1978). Although it is apparent that a fraction of extracellular ODN does enter cells in culture, the mechanisms involved are unclear, as is the quantity that gains entry (Uhlmann *et al.* 1990). Uptake is very much dependent on ODN charge, backbone chemistry and length as well as cell type (Noonberg *et al.* 1993) and experimental conditions (Iverson *et al.* 1992; Temsamani *et al.* 1994). Compositions of the extracellular medium, temperature and incubation period have also been found to influence cellular uptake (Vlassov *et al.* 1994).

Wickstrom *et al.* (1988) report an anti-c-*myc* unmodified ODN (PO-ODN) to inhibit *c-myc* protein expression in HL-60 cells in a dose dependent, sequence-specific manner. However, the studies were flawed in that the ODNs were radiolabelled at the 5'-end, and therefore highly susceptible to the action of 5'-end endonucleases which would cleave the radiolabel. Therefore the results may possibly have been a reflection of radiolabel trafficking and not the ODN. Although denaturing gel electrophoresis was used to monitor the presence of intact radiolabelled ODN, there is no quantitiative data to show the degree of degradation and therefore no measure of intact ODN in the cells. Electron micrographic studies by Zamecnik *et al.* (1994) showed PO-ODNs to be internalised into murine fibroblasts in an energy dependent manner, localising in the cytosol and, after 30-60 minutes, in the nucleus. These studies however involved ODNs labelled with radioisotopes (two different types) either at the end or internally and comparisons were made between three different cell lines. Binding profiles were reported for ODNs labelled with one type of isotope and electron micrographs from ODNs labelled with a different isotope. No account is taken of the influence of the radioisotope on binding or trafficking of ODNs, therefore conclusions cannot be drawn from these different experiments regarding PO-ODN binding and internalisation.

Intravesicular localisation of fluorescently labelled PO-ODNs was observed by Noonberg *et al.* (1993) in a number of different human cell lines, except for keratinocytes, therefore highlighting the cell specific nature of uptake. Caution must however be exercised regarding this data because the ODNs investigated by Noonberg *et al.* (1993) were composed of guanine and thymidine residues only, these two residues were shown be preferentially bound by cells in culture by Hughes *et al.* (1994). Therefore, this data cannot be used as a measure of the general ODN binding characterisitics to keratinocytes.

1.7.2 Uptake of Phosphorothioate Oligonucleotides

Following use of PS-ODNs in antisense experiments, a considerable number of studies investigated the cellular uptake characteristics of these ODNs *in vitro* (Loke *et al.* 1989; Iverson *et al.* 1992; Farrell *et al.* 1995) and *in vivo* (Saijo *et al.* 1994; Monia *et al.* 1996). Stein *et al.* (1988) observed the uptake of PS-ODNs to be slower than corresponding PO-ODNs in HL60 cells, which conflicted with the data obtained by Marti *et al.* (1989) who found no significant difference in uptake rates of both ODN types. Jaroszewski *et al.* (1991) attributed the discrepancies between ODN uptake data to a number of factors: differing cell systems, ODN lengths and type of label used, highlighting the caution that must be exercised when comparisons and correlations are made.

One fact that proves to be universal for PS-ODNs is that, compared to PO-ODNs, there is greater nuclease resistance and cellular association occurs to a higher degree. This latter phenomenon may, however, be attributable to the greater resistance to degradation and higher degree of binding to proteins, both factors which result in a higher final concentration intracellularly (Neckers *et al.* 1992). The greater anionic charge of PS-ODNs compared to PO-ODNs creates an ionic interaction with cationic proteins, thereby increasing non-specific interaction and binding (Beck *et al.* 1996).

1.7.3 Uptake of Methylphosphonate Oligonucleotides

The nature of MP-ODNs, a neutral backbone and greater hydrophobicity than other ODNs, predicts that they enter cells passively (Miller *et al.* 1981). Studies by Akhtar *et al.* (1991b) and Shoji *et al.* (1991) have shown MP-ODN uptake to be mediated by an endocytosis mechanism. Uptake was reduced at lower incubation temperatures (below 37°C) indicating an active energy requiring process, and binding was found to plateau after ten hours incubation indicating saturation (Shoji *et al.* 1991). The rate of MP-ODN uptake is also slower than that of PO- and PS-ODNs indicating different mechanisms (Zhao *et al.* 1993).

1.8 THE MECHANISMS OF OLIGONUCLEOTIDE INTERNALISATION AND INTRACELLULAR TRAFFICKING

The plasma membrane acts as a selective barrier to extracellular material. A number of mechanisms exist for the internalisation of exogenous compounds, the mechanism employed depends on size and charge of the particle to be internalised. In order to enhance ODN uptake and determine the intracellular fate of ODNs the mechanisms by which internalisation occurs must be understood. Extracellular material can be internalised into cells, for example by passive diffusion across the membrane, receptor mediated endocytosis (RME) or fluid phase endocytosis (FPE) (schematically represented in figure 1.5). Adsorptive endocytosis is yet another mechanism by which ODNs can be internalised by cells, this process is however random and non-specific. RME and FPE are the predominant mechanisms employed for ODN uptake.

1.8.1 Receptor Mediated Endocytosis

Specific receptors on the plasma membrane function to internalise exogenous material, which cannot gain entry passively, in a selective and controlled manner (Wileman *et al.* 1985). Many different receptors exist, binding and internalising

specific ligands. Once a receptor binds its ligand, this region of the plasma membrane invaginates and forms an intracellular vesicle, called a peripheral endosome. The process which follows is unclear, the peripheral endosome moves further into the centre of the cell and becomes larger, now being referred to as a perinuclear endosome. Some receptors recycle back to the surface at this stage in vesicles that fuse with the plasma membrane, others progress with the ligand into compartments known as endolysosomes which fuse or mature into lysosomes. Lysosomes contain a mixture of hydrolytic enzymes that function to degrade material that has been internalised by RME (Wileman *et al.* 1985; Alberts *et al.* 1989). The sequence of events are summarised in figure 1.6, endosomal and lysosomal compartments are discussed in more detail in section 5.1.1. The products of degradation are subsequently transported for use within the cell.

1.8.2 Fluid Phase Endocytosis (Pinocytosis)

The plasma membrane is continuously internalising proteins and lipids from its surface, regions of the membrane invaginate to form intracellular vesicles. Such invagination occurs constitutively (Wileman *et al.* 1985) or during RME (section 1.8.1). FPE, also known as pinocytosis, describes the process whereby exogenous fluid that becomes trapped in regions around invaginating plasma membrane, becomes internalised (Steinman *et al.* 1983). The rate of FPE is much slower than that of RME, and is not as selective or specific, occurring constitutively (Wileman *et al.* 1985). Cellular volume is maintained by exocytosis of the material in these vesicles. Substances such as sucrose and mannitol are internalised by FPE, and often used as markers for this mechanism (Besterman *et al.* 1981). Adsorptive pinocytosis describes the internalisation of molecules that are bound to the extracellular membrane during pinocytosis.

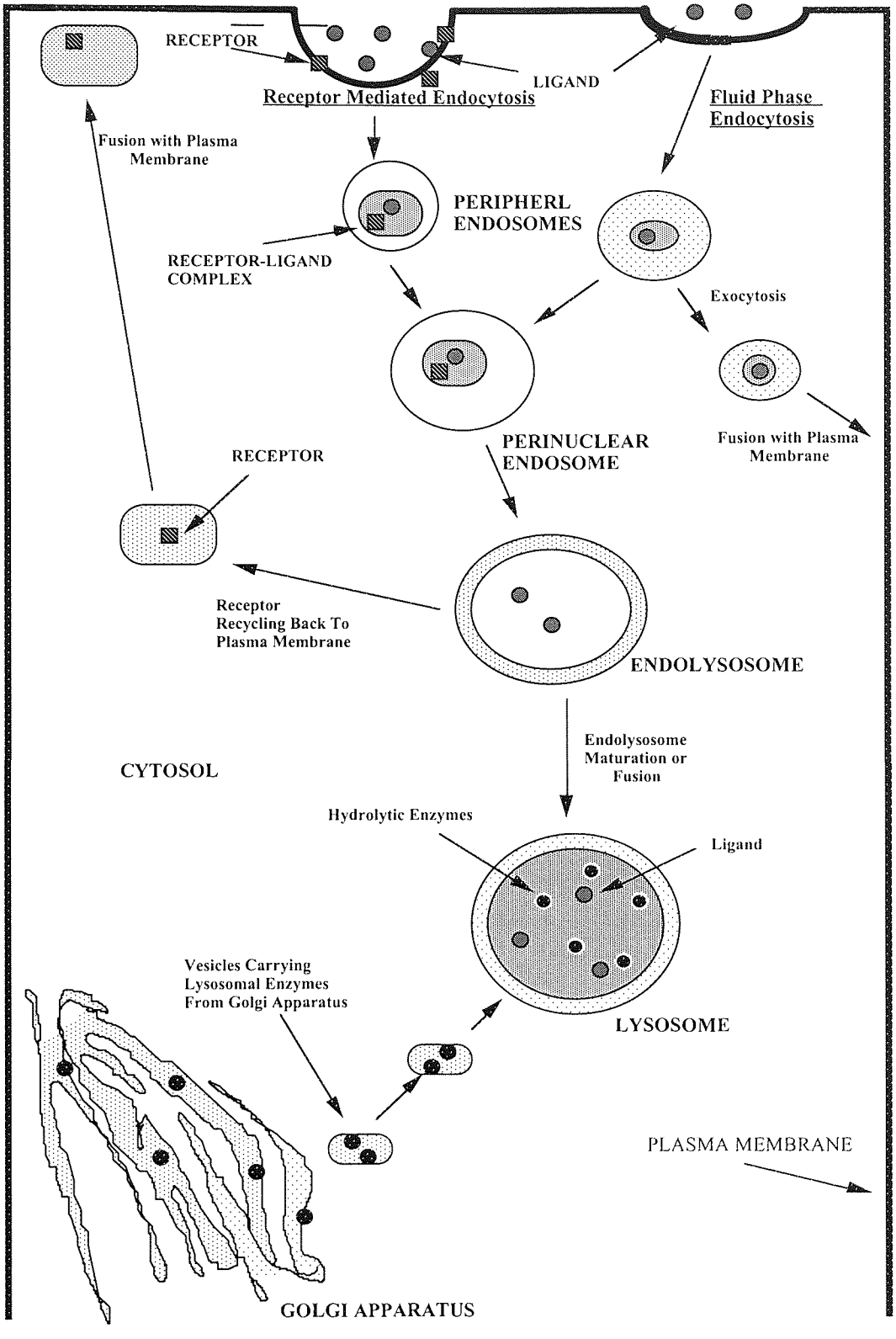


Figure 1.5: Schematic Diagram of Receptor Mediated Endocytosis (RME) and Fluid Phase Endocytosis (FPE). ODNs can either be internalised by natural uptake of exogenous material (FPE) or actively by binding to a surface receptor. Both routes appear to result in entrapment into intracellular vesicles, endosomes or lysosomes.

1.8.3 The Cell Surface Oligonucleotide Binding Proteins

A considerable amount of data obtained from studies of PO- and PS-ODNs cellular uptake, but not MP-ODNs, indicates the involvement of specific cell surface moieties in binding (Loke *et al.* 1989; Yakubov *et al.* 1989; Vlassov *et al.* 1994):- (1) Uptake is often found to be temperature dependent, diminished below an incubation temperature of 37°C, indicating the involvement of an active mechanism (Stein *et al.* 1988; Loke *et al.* 1989; Yakubov *et al.* 1989), (2) uptake is inhibited in the presence of metabolic inhibitors such as sodium azide (NaN_3) and 2-deoxyglucose (2DG) which inhibit cellular energy production (Yakubov *et al.* 1989; Gao *et al.* 1992; Wu Pong *et al.* 1994a), (3) there is a marked difference between the cellular uptake profiles of ODNs compared to those of fluid phase markers (Stein *et al.* 1993; Nakai *et al.* 1996), (4) ODN binding is sensitive to proteolytic washes, which remove cell surface proteins (Beck *et al.* 1996), (5) a limited binding capacity is implicated by cellular association profiles reaching a plateau after certain incubation periods (Yakubov *et al.* 1989; Iverson *et al.* 1992) and self competition studies revealing saturable binding (Beltinger *et el.* 1995; Beck *et al.* 1996), (6) cellular association is significantly increased at lower pH indicating an increase in surface protein binding (Goodarzi *et al.* 1991; Kitajima *et al.* 1992), and finally (7) the presence of agents that disrupt the process of endocytosis such as monensin (Wu Pong *et al.* 1994b) and phenylarsine oxide (Nakai *et al.* 1996) reduce uptake.

Loke *et al.* (1989) identified a putative plasma membrane nucleotide-binding protein, 80kDa in size, in HL60 cells, two membrane nucleotide-binding proteins, 79kDa and 90kDa, were identified by Yakubov *et al.* (1989) in mouse fibroblasts, and both Akhtar *et al.* (1996) and Hawley *et al.* (1996) detected a 46kDa protein which bound both PO-ODNs and PS-ODNs in the membrane of an epithelial carcinoma cell line and fibroblasts respectively. Beltinger *et al.* (1995) detected the presence of a number of different putative ODN binding proteins of varying size. These putative nucleotide-binding proteins (reviewed by Vlassov *et al.* 1994) probably account for the unexpected uptake of polyanionic PO-ODNs across the membrane. The natural function of these proteins is currently unknown. Bennett *et al.* (1988) propose these

nucleotide-binding proteins as receptors functioning to salvage and recycle circulating nucleotides. Vlassov *et al.* (1994) conclude that the mechanism of internalisation to be that of endocytosis. Despite the presence of these receptors, ODN uptake is not very efficient. More recently Bijsterbosch *et al.* (1997) describe PS-ODN binding to rat liver cells by a putative scavenger receptor which has a broad specificity, binding polyanionic molecules such as lipo-proteins, polynucleotides and polysaccharides. Scavenger receptors may account for ODN uptake by liver cells, and possibly other cell lines aswell. Mac-1, a cell surface receptor found on polymorphonuclear leukocytes, macrophages and natural killer cells, which binds fibrinogen and intracellular adhesion molecule amongst other natural ligands, also bind and internalise ODNs (Benimetskaya *et al.* 1997). There is obviously a great variation in the proteins between cell lines that will need further investigation if they are to be targets for antisense agents therapeutically.

1.8.4 Intracellular Fate of Oligonucleotides

Regardless of whether ODNs are internalised by FPE or RME, it appears that they become encapsulated into intracellular vesicles such as endosomes or lysosomes. A number of studies, which have tagged ODNs with fluorescent labels, have shown intracellular distribution to adopt a punctate pattern indicative of intravesicular distribution (Noonberg *et al.* 1993; Shoji *et al.* 1996). PO-ODNs were shown to distribute in a punctate pattern by Noonberg *et al.* (1993) in human keratinocytes, as were PS-ODNs in vero cells (Shoji *et al.* 1996). Stein *et al.* (1984) also found evidence for endosomal/lysosomal distribution of PS-ODNs in HL60 cells, finding that disrupting the low pH within these compartments lead to ODN release into the cytoplasm as indicated by an increase in fluorescence intensity. However, Stein *et al.* (1984) presented data demonstrating different trafficking pathways of PO-ODNs and PS-ODNs. PS-ODNs were found to be encapsulated into acidic vesicles, either endosomes or lysosomes where they appeared to become trapped, residing in the cells for a longer period of time than PO-ODNs (Stein *et al.* 1984). PO-ODNs were located in the cytoplasm, nucleus or were exocytosed (Stein *et al.* 1984).

A major problem with the endocytosis of ODNs is their entrapment in endosomal or lysosomal compartments. ODNs must escape these compartments into the cytoplasm before lysosomal degradation and in order to reach their targets (Leonetti *et al.* 1993). If the lysosomal compartments cannot be bypassed, the stability of ODNs must be prolonged for a period long enough to allow escape into the cytoplasm. Little data is available regarding ODN stability in lysosomes. Hudson *et al.* (1996a) reported PO-ODNs to have a $t^1/_2$ of only 30 minutes in a lysosomal extract, compared to PS-ODNs which had a $t^1/_2$ of 90 minutes. The modification of ODN by their backbone chemistry and the conjugation of groups at either end in order to increase stability to exogenous nucleases should also confer stability to degradation by lysosomal enzymes. Therefore study of ODN stability within lysosomes is essential.

1.9 OLIGONUCLEOTIDE DELIVERY STRATEGIES

As discussed in section 1.8.3, endocytosis is probably the method by which cells internalise ODNs, although efficiency is poor. From a pharmacological aspect, ODN uptake/penetration into cells has to be enhanced so as to increase intracellular bioavailability. Modifications have been made to the phosphodiester backbone of ODNs to increase resistance to nuclease degradation (section 1.6). A similar approach has been taken to enhance cellular delivery and bioavailability, by the modification of ODN chemistry, attachment of conjugate moieties, and use of specific delivery methods (reviewed by Budker *et al.* 1992; Jaroszewski *et al.* 1991; Vlassov *et al.* 1994). Many methods have been studied with respect to this aim, some are reviewed below.

1.9.1 Microinjection and Electroporation

The process of microinjection involves the direct insertion of an antisense agent into the cell cytoplasm, therefore bypassing encapsulation into endosomal/lysosomal compartments (Leonetti *et al.* 1991). Studies have shown ODN localisation in the

nucleus following microinjection into the cytoplasm (Leonetti *et al.* 1991; Fisher *et al.* 1993), which is ideal if pre-mRNA or the gene itself were the target. The mechanism by which ODNs are trafficked into the nucleus is poorly understood. Leonetti *et al.* (1991) found the process to be energy independent, thus indicating passive diffusion as the mechanism involved. Microinjection data may prove useful because the path followed by microinjected ODNs may mimic the events that follow endosomal/lysosomal release into the cytoplasm. Woolfe *et al.* (1990) showed mRNA degradation due to RNase-H activity following microinjection of a PS-ODN in Xenopus oocytes. These studies therefore demonstate the potential of this delivery method for ODN delivery. However, the major drawback of microinjection is the single-cell basis of the method, restricting its use. The use of this method may therefore have to be restricted for use as a research tool, as opposed to a therapeutic use.

Electroporation is another method whereby ODNs can be directly inserted into cells. Cells are subjected to a high voltage in serum free medium containing the ODN (Bergan *et al.* 1993). Intracellular ODN concentrations were found to increase between 3-fold to 27-fold and uptake was dependent on cell or ODN type (Bergan *et al.* 1993). As with microinjection, ODNs are mainly localised in the nucleus.

Bergan *et al.* (1993) found suppression of the *c-myc* gene following electroporation of U937 cells in medium containing an anti-myc ODN. Cells were electroporated in the presence of fluorescein labelled PS-ODNs and PO-ODNs. Cell associated fluorescence was found to be elevated 1hr after electroporation for both backbone types, acid washing confirming this elevation was not due to an increase in cell surface binding (Bergan *et al.* 1993). Levels of the c-myc protein were assessed relative to its short half life, there being almost a total absence of the protein 24hrs after electroporation when a 50μM concentration of ODN was used. Bergan *et al.* (1993) did not, however provide any information as to intracellular ODN stability following electroporation. Therefore, the decrease in c-myc protein levels may not be due to a true antisense effect as exerted by the antisense ODN.

1.9.2 Increasing Oligonucleotide Hydrophobicity/Lipophilicity and Conjugation to Poly (L)-Lysine

One of the simplest methods suggested to increase cellular ODN delivery is to make the structure more lipophilic (Akhtar *et al.* 1992a; Budker *et al.* 1992). A number of lipophilic molecules have been investigated. The effect of conjugating cholesterol (section 4.1.2.1), poly-ethylene glycol (section 4.1.2.2), and alkyl chain (section 4.1.2.3), to improve ODN uptake is discussed later.

Leonetti *et al.* (1993) proposed a non-specific RME pathway of cellular uptake, PLL-ODNs are found to accumulate in acidic compartments and were affected by metabolic inhibitors. Lemaitre *et al.* (1987) found inhibition of the Vesicular stomatitis virus (VSV) following treatment of infected cells with a poly (L)-lysine (PLL) conjugated ODN. PLL has been used as a DNA binding moiety (Wagner *et al.* 1990 and 1991) due to its affinity for DNA and ODNs. The problem with PLL is its cellular toxicity, and the possibility of self binding which would impede membrane binding. Leonetti *et al.* (1993) observed PLL-ODNs to be 10- to 50-fold more active as anti-VSV agents than unconjugated ODNs. Also observed was the efficient anti-proliferative activity of anti-*c-myc* -PLL-ODNs in the presence of serum containing culture medium, non-conjugated ODNs were found to be ineffective due to nuclease degradation (Degols *et al.* 1991). More recently Goto *et al.* (1997) found that an antisense ODN to interleukin-1 beta, conjugated to PLL, exhibited both *in vitro* and *in vivo* dose-dependant and sequence specific inhibition of production of this protein.

1.9.3 Liposomal Delivery

Liposomes are microscopic spheres of artificial lipid bilayers which can be prepared in various sizes. Liposomes were originally used as a model for the plasma membrane, however the ability to encapsulate compounds in the aqueous interior or insert molecules into the hydrocarbon regions of the bilayer has lead to their use as a delivery vehicle (Akhtar *et al.* 1991b). Liposomes are biodegradable, biocompatible,

easily modified. Liposomes are able to deliver encapsulated ODNs irrespective of their natural ability to cross the plasma membrane, provide ODNs with protection from nuclease degradation and provide the potential to produce sustained and controlled release formulations (Akhtar *et al.* 1992b). Delivery is achieved by fusion of liposomes with the membrane (Budker *et al.* 1992; Vlassov *et al.* 1994). Akhtar *et al.* (1992b) found saturable binding of a PS-ODN to liposomes, followed by relatively slow release, efflux half life ($t^1/_2$) of 6 to 9 days compared to 24 hours for a marker molecule, glucose. ODN entrapment into liposomes varied, the amount being dependent on ODN type, lipid formulation and method used to prepare the liposomes (Akhtar *et al.* 1992b). MP-ODNs have been found to have the greatest capacity to bind liposomes due to their greater hydrophobicity, followed by PS-ODNs, both greater than PO-ODNs (Akhtar *et al.* 1992b).

A number of different types of liposomes can be prepared, depending on morphology and lipid composition cationic, pH-sensitive, immunoliposomes and Sendai virus-derived liposomes (reviewed by Akhtar *et al.* 1992b and Rojanasakul *et al.* 1996).

1.9.3.1 Cationic Liposomes

Cationic liposomes are prepared from cationic lipids such as N-(1-(2,3-dioleyloxy)propyl)-N,N,N-tri-methylammonium chloride (DOTMA), which was found to bind most efficiently to polyanionic molecules such as ODNs, RNA or DNA by electrostatic interaction (Jaaskelainen *et al.* 1994). A number of studies show enhanced cellular ODN delivery using cationic liposomes (Bennett *et al.* 1992; Thierry *et al.* 1993; Lappalainen *et al.* 1994). Thierry *et al.* (1993) found complete inhibition of P-glycoprotein expression in cells treated with an antisense ODN delivered by cationic liposomes compared to only a 40% reduction by liposome free ODNs.

Bennett *et al.* (1992) found PS-ODNs delivered by cationic liposomes to localise in both the nucleus and discrete cytoplasmic structures, indicative of intracellular vesicles as observed by punctate fluorescent distribution. Liposome free ODNs

localised in these discrete cytoplasmic structures only. A 1000-fold increase in antisense effect was observed for a PS-ODN in a cationic liposomal preparation, but only a 6- to 15-fold increase in cellular association was observed compared to the absence of liposomes (Bennett *et al.* 1992). All assessments were made after a 4hr incubation period between the cells and ODN. Degradation of the ODN in the extracellular medium was not investigated following this incubation period. Therefore the increases in cellular association may have been as a result of the binding of ODN fragments or label only to the cells, and not intact ODN. Bennett *et al.* (1992) stated that cationic liposomes could be used to efficiently deliver ODNs to the nucleus, for targetting pre-mRNA or a gene. However, lack of stability data does not enable any real conclusions to be made regarding ODN delivery, the results of this study may have reflected the distribution of degraded fluorescently labelled ODN.

In contrast to the report by Bennett *et al.* (1992), Lappalainen *et al.* (1997) found ODNs delivered by cationic liposomes to internalise into the intravesicular compartments of cells, from which they were released into the cytoplasm and trafficked to the peri-nuclear region, following 4hrs incubation. A mechanism of endocytosis of the ODN encapsulated in the cationic liposomes was implicated by this study, due to the pattern of intracellular localisation. A critisism of this study, as with Bennett *et al.* (1992), is that degradation of the ODN was not investigated, therefore the trafficking patterns may have due to label or ODN fragments.

Studies into the intracellular trafficking of liposome encapsulated ODNs appear to vary in terms of the patterns of distribution observed between cell types. The nature of the liposomal preparation used, cell line and type of ODN label probably influence this distribution (Lappalainen *et al.* 1997).

Wielbo *et al.* (1997) reported the decrease in levels of angiotensinogen mRNA and protein following antisense PS-ODN delivery via cationic liposomes, in a dose dependant manner. Rat hepatoma cells were treated with the cationic liposome complexed with ODNs and control empty liposomes. A 90% expression level of angiotensinogen protein was found for cells treated with empty liposomes compared

to only 22% for cationic liposome-ODN complexes, 70% expression observed for cells treated with naked ODNs.

1.9.3.2 Immuno-Liposomes

In an aim to increase specificity, immuno-liposomes were developed, enabling the targetting of specific receptors. Liposomes were conjugated to an antibody to or specific ligand of a cell surface receptor (Leonetti *et al.* 1990). Over 95% reduction in VSV replication was observed in murine cells treated with antibody-targetted liposomes (immuno-liposomes) containing an anti-VSV ODN, empty or random-sequence ODNs loaded liposomes conferring no change in viral replication (Leonetti *et al.* 1990). Zelphati *et al.* (1994) report inhibition of HIV replication in chronically infected cells following treatment with immuno-liposomes encapsulating an anti-HIV PS-ODN.

1.9.3.3 pH-Dependent Liposomes

As discussed, studies of liposomal delivery indicate the involvement of an endocytic pathway, resulting in liposome entrapment in endosomal and lysosomal compartments (Bennett *et al.* 1992). Liposomes that were sensitive to the pH change in acidic endosomal compartments were formulated. These liposomes were designed such that they released their contents into the cytoplasm prior to fusion with lysosomes, bypassing the problem of degradation within lysosomes. Ropert *et al.* (1992) and De Oliveira *et al.* (1997) report a greater antisense ODN efficiency of pH-sensitive liposomes compared to non-pH-sensitive liposomes in the inhibition of Friend leukemia virus, the former enhancing cytoplasmic delivery. Free antisense ODNs, to the *env* mRNA, showed no antiviral activity in virus infected cells compared to ODNs conjugated to pH-sensitive liposomes (De Oliveira *et al.* 1997). De Oliveira *et al.* (1997) also found that cells undergoing viral efflux would have a more rapid uptake of liposomes, this being attributed to an increase in pinocytosis, therefore aiding the uptake process.

1.9.4 Oligonucleotide Conjugation to a Receptor Ligand

The process of RME can be utilised for delivering ODNs to cells by targetting specific cell surface receptors. A number of other receptors have been targetted for ODN delivery, some are listed in table 1.2.

ODNs can be conjugated to the ligands of these receptors, with the aim of internalisation of the ligand-ODN complex by RME (reviewed by Rojanasakul *et al.* 1996). Section 3.1.7 and 3.1.8 discuss targetting of the mannose receptor with mannosylated-ligands and mannosylated-ODNs respectively, as means of enhancing cellular delivery.

In all the cases (table 1.2) receptor ligand conjugated ODNs had a greater antisense effect compared to unconjugated control ODNs. Wu *et al.* (1992) observed inhibition of Hepatitis B viral gene expression in cells treated with an antisense ODN-asialoorosomucoid conjugate, targetting the asialoglycoprotein receptor and Citro *et al.* (1992) found transferrin conjugated anti-c-*myc* ODN, targetting the transferrin receptor of HL60 cells, inhibited proliferation following RME of the conjugate. In 1993, Midoux *et al.* successfully transfected HepG2 cells with glycosylated plasmid DNA targetted to the galactose receptor, and Hangeland *et al.* (1997) targetted the galactose receptor of HepG2 cells with a MP-ODN bearing a glycoprotein residue which was a ligand for the receptor, *in vivo*. Hangeland *et al.* (1997) revealed the greatest disposition of tail vein injected ODNs in the liver and kidney, the mechanism by which the complex gained entry into the cell as endocytosis. The galactose receptor of HepG2 cells was targetted by Roth *et al.* (1997) by a PO-ODN conjugated to a asialoglyco-protein by PLL. The antisense ODN was found to downregulate of its target protein more efficiently when complexed to the receptor ligand by 20%, and also decrease the effective ODN dose.

Table 1.2: Receptor Ligand Conjugated Oligonucleotides for Enhancing Delivery.

LIGAND	RECEPTOR	Cell Line	REFERENCE
Asialooroso-muciod	Asialoglyco-protein	HepG2	Wu *et al.* (1992)
Transferrin	Transferrin	HL60	Citro *et al.* (1992)
Mannosylated Protein	Mannose	J774E	Bonfils *et al.* (1992)
6-Phospho-mannose-BSA	6-Phospho-mannose	J774E	Bonfils *et al.* (1992)
Glysosylated DNA	Galactose	HepG2	Midoux *et al.* (1993)
Folic Acid	Folic Acid	HL60	Citro *et al.* (1994)
Transferrin Receptor Antibody	Transferrin Receptor	U87, ECV40	Walker *et al.* (1995)
Synthetic ASGP ligand/MP-ODN Conjugate	Asialoglyco-protein (ASGP) Receptor	HepG2	Hangeland *et al.* (1997)
Synthetic ASGP ligand/PLL/PO-ODN Conjugate	Asialoglyco-protein (ASGP) Receptor	HepG2	Roth *et al.* (1997)

Gedda *et al.* (1997) have shown the enhanced targetting and uptake of double stranded DNA conjugated to EGF in human glioma cells, further supporting the potential for ODN targetting to this receptor. EGF is a potent mitogenic peptide which initiates a cascade of intracellular responses such as DNA synthesis, cellular proliferation and motility (Khazaie *et al.* 1993). Approximately 48% of malignant human tumours possess an amplified EGFr gene. A431 cells, vulval epidermoid carcinoma, express 10- to 50-fold higher levels of EGF-receptor than other cell lines, this high level of expression of this gene is implicated in cellular transformation (Ullrich *et al.* 1984).

In a slightly different approach, Walker et al. (1995) conjugated transferrin receptor antibodies to an ODN in order to enhance ODN cellular delivery. Systems employing transferrin for ODN delivery were found to compete for natural transferrin conjugated proteins, therefore impeding natural cellular processes. Therefore the approach of using antibodies to the receptor was assessed. Walker et al. (1995) found approximately a 3-fold increase in cellular association for ODNs conjugated to the transferrin receptor antibody in human and endothelial cell lines.

Although the above reports (table 1.2) support the use of RME as a means of enhancing ODN uptake, amongst many others, there is one major problem with this approach. RME is followed by entrapment in intracellular vesicles, therefore necessitating an escape mechanism by the drug. As discussed in section 1.8.4, the intracellular fate of ODNs is unclear. Regardless of such entrapment, the above studies show the ODNs, delivered by RME, have a positive inhibitory effect, indicating that such escape mechanisms may not be crucial. The fate of ODNs in part is determined by the fate of receptor to which the ODN was targetted. Receptors have different fates following endocytosis, they can be disassociated from their ligand and recycled back to the surface like mannose or LDL receptors, or degraded in lysosomes with their ligand as occurs for the receptor for IgA (Wileman *et. al.* 1985). Following uptake enhancement of ODNs by RME, the problem of intracellular bioavailability needs addressing.

1.9.5 Sustained Delivery of Oligonucleotides

Biological instability of ODNs to extra- and intra-cellular nucleases, poor cellular uptake, low rate of delivery to the actual target site and rapid elimination kinetics *in vivo* have given rise to the development of devices that repeatedly release the ODN to the target. These are termed sustained delivery devices. A constant controlled infusion of ODN to the target site eliminates the requirement for repeated delivery and compensates for the above limiting factors, being particulary useful for long term therapy.

1.9.5.1 Microspheres

Microspheres are spherical, microscopic, matrices of polymer. Drug compounds are dispersed within the polymer matrix, and released slowly to the exogenous medium. Therefore, like liposomes, microspheres encapsulate drug molecules and are a means of ODN delivery across cell membrane. Akhtar *et al.* (1997) studied poly(lactide-co-glycolide) microspheres, which are biodegradable, for delivery of PO-ODNs and PS-ODNs to a macrophage cell line, finding a 10-fold increase in cellular association of the ODNs following encapsulation, decreasing in the presence of metabolic inhibitors. Intracellular trafficking studies revealed a diffuse pattern of fluorescence labelled microsphere-ODNs indicating localisation in the cytoplasm compared to a punctate distribution of fluorescence for the control ODN, indicative of localisation within intracellular vesicles (Akhtar *et al.* 1997). Microspheres are phagocytosed into cells, it remains unclear whether they are internalised into intracellular veiscles or the cytoplasm or a combination of the two. Therefore, for targetting mRNA in the cytoplasm, microspheres appear to offer an attractive means of ODN delivery, bypassing internalisation into endosomes and lysosomes. However, further studies are required to verify localisation of microsphere-ODN complexes in the cytoplasm, and therefore ability to reach the target site.

1.9.5.2 Polymer Matrices

Nucleic acids can also be entrapped within polymeric matrices, from which they are released in a predictable and controlled manner (Lewis *et al.* 1995; Cleek *et al.* 1997). Polymer systems for the release of drugs have been under study for over twenty years (Heller *et al.* 1993). Different polymers have different properties, some toxic and some non-toxic. Polymers have the potential to provide ODNs with protection from degradation by nucleases (Lewis *et al.* 1995; Hudson *et al.* 1996b). Polymers have been developed for use as sustained release systems, implanted at the desired site for drug release, those that are biodegradable are themselves degraded into biocompatible products following release. Lewis *et al.* (1995) studied ODN release from poly (L-lactic acid) matrices, PS-ODN release was found to be slow compared to PO-ODN, ODNs of shorter length to be released faster, a biphasic release profile for all ODNs, a burst of release during the first 48hours followed by slow sustained release, resistance to nuclease degradation and no affect on hybridisation capacity to their targets. Scanning electron microscopy and differential scanning calorimetry analysis of the poly (L-lactic acid) matrices revealed no significant degradation (Lewis *et al.* 1995). Hudson *et al.* (1996b) found similar release characteristics for ribozymes from poly (L-lactic acid) matrices, and that biological stability increased from 2 seconds for free ribozymes to 2 weeks for polymer entrapped ribozyme. Both studies highlight the potential for using polymer matrices for the sustained release of antisense agents and the improvement of stability. Dose dependant growth inhibition of smooth muscle cells in rats was observed by Cleek *et al.* (1997) following the release of ODNs from poly (D L-lactic-co-glycolic acid) polymers. Films containing entrapped ODNs were placed in the cell culture wells along with the cells during the experiment. The ODNs were antisense to a glycoprotein, tenascin, that regulates migration and differentiation of smooth muscle cells (Cleek *et al.* 1997). ODN entrapment was approximately 74% efficient, and release found to follow a biphasic pattern similar to that found by Lewis *et al.* (1995). Cleek *et al.* (1997) did not however, provide any data as to stability or the uptake of ODN by the cells, nor was any data regarding the levels of tenascin mRNA or protein provided. Therefore, although it appeared as if proliferation was inhibited, the mechanism by which it was occuring was not clear. Details of the

stability of these polymer devices in the presence of biological material and surface release due to erosion is essential, as are the kinetics of ODN uptake and subsequent intracellular activity.

1.9.5.3 Nanoparticles

The use of nanoparticles for the delivery and release of ODNs has received significant attention (Chavany *et al.* 1992 and 1994; Godard *et al.* 1995; Zobel *et al.* 1997). Nanoparticles are colloidal particles, those used for ODN delivery are often composed of cationic macromolecules such as polyhexylcyanoacrylate (Zobel *et al.* 1997) which are able to adsorb or entrap ODNs. Chavany *et al.* (1994) found nanoparticles to protect ODNs from 5'-end exonuclease degradation by 39%, and increase cellular uptake by 8-fold compared to free ODNs. Zobel *et al.* (1997) entrapped PO-ODNs in nanoparticles, finding these ODNs to be resistant to degradation from the endonuclease DNase-1, a 20-fold increase in cellular uptake *in vitro* and intravesicular localisation of fluorescein labelled ODNs. An endocytic uptake process for ODN-nanoparticles was hypothesised by Zobel *et al.* (1997) following diffuse and punctate patterns of fluorescence for ODNs/nanoparticles associated during microscopic studies. Little or no fluorescence for free ODNs was observed in this study by Zobel *et al.* (1997). The improvements in ODN stability and enhancement of cellular uptake following ODN adsorption onto nanoparticles as reported by Chavany *et al.* (1994) and Labhasetwar *et al.* (1997) highlight the potential of this delivery method for therapeutic use, the latter report describing the application of nanoparticles for drug delivery for inhibiting restinosis. Further intracellular trafficking studies are required to pinpoint the exact location of the ODNs/nanoparticle complexes once internalised as are the release kinetics of the ODNs from these particles, neither study presenting any release data.

1.9.6 Other Methods for Oligonucleotide Delivery

1.9.6.1 Viral Envelopes

A number of other alternatives exist for ODN delivery. Using a similar concept to liposomes, the use of 'viral envelopes' has been developed (reviewed by Uhlmann *et al.* 1990; Budker *et al.* 1992; Vlassov *et al.* 1994). Virions infect host cells by fusion with the plasma membrane, a process which is mediated by their membrane. This fusion mechanism was exploited for delivery to cells by the encapsulation of ODNs. Viral envelopes are advantageous due to their biodegradable, biocompatible nature, uptake ability and protection of their contents from degradation (Vlassov *et al.* 1994). The major advantage of viral envelopes is the ability to deliver their contents into the cytoplasm, by-passing any degradation in lysosomes as with liposomal delivery. Study of reconstituted envelopes of Sendai virus (RESV) revealed approximately 30% ODN entrapment and successful inhibition of viral replication by anti-viral ODNs loaded in RESV (Vlassov *et al.* 1994).

1.9.6.2 Retroviral Vectors

Retroviruses, like viral envelopes, can be used for cellular delivery by the same principles, fusion with host cell membrane and insertion into the cytoplasm (reviewed by Reddy, 1996). Retroviruses are engineered such that they do not possess any pathogenic viral material, and are therefore unable to replicate, but however are able to internalise into host cells. Phillips *et al.* (1997) delivered an ODN, antisense to the mRNA of the angiotensinogen receptor protein, using an adenovirus-associated vector. Downregulation of receptor expression was found *in vitro* when the viral vector was used for ODN delivery and *in vivo* the blood pressure of hypertensive rats was decreased following injection of the viral vector delivered ODNs to the brain.

1.9.6.3 Erythrocyte Ghosts

Human erythrocyte membrane preparations, termed erythrocyte ghosts, have been developed as ODN carriers (reviewed by Budker *et al.* 1992; Vlassov *et al.* 1994). Erythrocytes subjected to hypo-osmotic shock will lyse, releasing free intact membrane which can be manipulated to encapsulate ODNs. Grimaldi *et al.* (1997) reported that cellular uptake of anti-HIV ODNs was enhanced by the erythrocyte ghost delivery method, resulting in successful inhibition of viral growth *in vitro* .

1.9.6.4 Fusogenic Peptides

As a means of increasing cytoplasmic delivery of ODNs, fusogenic peptides, derived from regions of viral envelope protein have been investigated. Fusogenic peptides usually span a 30 amino acid region of envelope protein, and comprise of alternating hydrophillic and hydrophobic domains. These domains undergo conformational changes at low pH, which allow their interaction with the lipid portion of intravesicular membranes, such as endosomes, and are thought to facilitate fusion with and release from these membranes into the cytoplasm (Bongartz *et al.* 1994; review by Rojanasakul *et al.* 1996: Wyman *et al.* 1997). A 5- to 10-fold increase in anti-HIV activity was found by Bongartz *et al.* (1994) following conjugation of an anti-HIV ODN with a fusogenic peptide in infected lymphocytes also reporting endosomal efflux and a diffuse cytoplasmic pattern of labelled ODNs following fusogenic peptide conjugation, compared to a control unconjugated ODN.

The hydrophobic fusion domain of HIV gp41 and the hydrophillic domain for SV40 nuclear localisation sequence were conjugated and this peptide complexed with a PS-ODN by Morris *et al.* (1997). The resulting peptide/ODN complex was found to efficiently enter human fibroblast cells in less 1hr, as assessed by fluorescent microscopy, Morris *et al.* (1997) report over 90% of the ODN dose delivered. Following delivery, intracellular localisation was mainly nuclear compared to intra-vesicular localisation for control ODNs, in agreement to the reports by Bongartz *et al.* (1994). Morris *et al.* (1997) also found that conjugating the ODN to the peptide

increased ODN stability to serum nuclease degradation, attributed to the formation of a particle comprising of peptide surrounding the ODN. Both these studies demonstrate the potential of fusogenic peptides as a delivery method for ODNs, once delivery to the cytoplasm has been achieved there is the potential for trafficking to the nucleus.

1.9.6.5 Cyclodextrins

Cyclodextrins are cyclic structures comprising of glucose polysaccharides, arranged such that there is a cavity into which molecules can be encapsulated, such as ODNs (Zhao *et al.* 1995). A 2- to 3-fold increase in uptake of encapsulated PS-ODN is reported along with no effect on ODN stability or efflux, fluorescein labelling showing intracellular localisation (Zhao *et al.* 1995). The viral activity of a PO-ODN was found to be enhanced by delivery involving cyclodextrins as reported by Abdou *et al.* (1997). A 90% inhibition of viral growth was found for cyclodextrin delivered ODNs compared to on average 23% for naked ODNs, the effective dose also reduced by this delivery method.

1.9.6.6 SupraMolecular BioVector

Berton *et al.* (1997) reports an alternative delivery vehicle for ODNs, in order to improve cellular uptake. The SupraMolecular BioVector (SMBV) is a multi-layered particle composed of cross-linked polysaccharides surrounded by fatty acids and a lipid layer, the structure being able to entrap molecules within its core. Aside from ODNs, a number of other molecules have been entrapped for example doxorubicin, gentamicin and interleukin-2. An 8-fold increase in stability of ODN/SMBV complex in culture medium was observed and uptake was decreased at 4°C incubation and by sodium azide, a metabolic inhibitor which depletes cellular energy levels (Berton *et al.* 1997). Studies involving a fluorescein conjugated ODNs revealed intravesicular distribution, within endosomes or lysosomes, of ODN-SMBV complexes, however, compared to control ODNs, there was an 11-fold increase in cytoplasmic localisation without any detectable toxicity (Berton *et al.* 1997). Data for ODN release from the

ODN-SMBV complexes is lacking however. Therefore, although stability, uptake, and intracellular localisation data highlights the potential of this method as a delivery mechanism, efficient release profiles will be essential. Studies continue on use of SMBVs for ODN delivery.

1.10 OLIGONUCLEOTIDE STUDIES *IN VIVO*

Following *in vitro* study, ODNs have to be assessed *in vivo* if they are to be developed for therapeutic use. As with studies *in vitro,* a vast number of factors have to be taken into consideration before conclusions and correlations can be made as to ODN efficacy, such as ODN chemistry, disease, animal model and delivery method. PS-ODNs have been most extensively studied *in vivo*. Srinivasan *et al.* (1995) and Akhtar *et al.* (1997) review the *in vivo* pharmacokinetics of ODNs. Some *in vivo* studies are listed in table 1.3, highlighting the range of different ODN targets, animal systems to which they can be applied and the results of these studies.

A number of facts have emerged from *in vivo* pharmacokinetic studies into ODN therapy:- (1) ODNs are generally localised in the organs of the reticuloendothelial system, liver, kidney, spleen and lungs (Saijo *et al.* 1994; Butler *et al.* 1997; DeLong *et al.* 1997). These cells are ideal targets for ODNs conjugated with certain carbohydrates or glycoproteins because they bear the corresponding receptors. Saijo *et al.* (1994) found the liver and kidney to display the highest concentrations of intra-peritoneally dosed PS-ODNs, the lowest concentrations were found in the brain where there were no detectable ODN levels after 48hrs compared to the initial levels in the liver and kidney (Butler *et al.* 1997). Immunohistochemistry, fluorescence microscopy and autoradiography revealed the same pattern of distribution and intensity of signal, the majority of the ODN intact in the liver and kidney. DeLong *et al.* (1997) performed *in vivo* distribution and stability studies of PS-, PS_2- and MP-ODNs in nude mice, induced to form human pancreatic tumours. The liver and kidney were found to be the main sites for accumulation, and the volume of distribution in the order PS > PS_2 > MP-ODN.

Table 1.3: Oligonucleotide Studies *In Vivo.*

Animal Model	ODN Target	Method of Admin.	Results	Reference
Human Dermal Tumour Xenografts in Mice	PO-ODN to N-*myc*	Micro-osmotic Pump	50% Tumour mass Reduction and Decrease in *myc* Protein Level	Whitesell *et al.* (1991)
Fibroblastic tumours in mice	PO/PS-chimeric ODN to NF-κB Transcription Factor	Intra-peritoneal Injection	Reduction in tumour size	Kitajima *et al.* (1992)
Human Fibrosarcoma Xenografts in nude mice	PS-ODN to NF-κB Transcription Factor	Sub-cutaneous Injections or osmotic pump	Over 70% reduction in tumour size	Higgins *et al.* (1993)
LOX Ascites Tumour in Mice	PS-ODN to p120 protein	Intra-Peritoneal Injection	ODN stable up to 48hrs post injection.	Saijo *et al.* (1994)

Rats with Intracerebral Tumours	PS-ODN to Urokinase protein	Intra-tumour catheter	No detectable ODN toxicity.	Engelhard *et al.* (1996)
HumanTumour Xenografts in Mice	PS-ODN to c-*raf* Kinase gene	Intra-venous Injection	Inhibition of Tumour Growth and decrease in c-*raf* mRNA	Monia *et al.* (1996)
Nude mice implanted with Prostate tumours	PS-ODNs to transforming growth factor.	Sub-cutaneous diffusion pumps	No hisotological evidence of toxicity	Rubenstein *et al.* (1997)
Normal rats	PO-ODNs in liposomes conjugated to Sendai-virus	Single Aerosol delivery	47% delivery to trachea, 39% delivery to bronchi.	Yonemitsu *et al.* (1997)
Normal rats and BALB/c mice	PS-ODNs to human Papillomavirus	I.V.	Similar distribution of ODN in tissues - mainly liver and kidney.	Butler *et al.* (1997)
Nude mice bearing human pancreatic tumours.	PO-, PS- and MP-ODNs to the K-ras oncogene	Tail vein injection.	Similar tissue/tumour distribution, for PO-, PS- and MP-ODNs	DeLong *et al.* (1997)

(2) Single ODN doses having the desired positive antisense effect are rare, and continued infusion/administration is often required for biological efficacy. Rapid distribution and elimination kinetics of ODNs delivered systemically results in the need for repeated systemic delivery, which is costly. Rapid degradation of the ODNs may give rise to the potential for the incorporation of modified nucleotides into cellular DNA leading to mutagenicity. Therefore, sustained release devices appear ideal for ODN delivery. A 50% decrease in tumour mass was observed by Whitesell *et al.* (1991) following a continuous 2 week ODN infusion by a micro-osmotic pump, no toxicity being observed during this time period. (3) Desired effects are sometimes obtained without knowing the precise ODN mechanism of action, sequence-specific but not antisense (Coulson *et al.* 1996). Rockwell *et al.* (1997) report the non-sequence specific binding of a PS-ODN to cell surface expressed receptors epidermal growth factor receptor and flk-1, interfering with normal binding of their ligands and thus ligand-mediated receptor phosphorylation. Rockwell *et al.* (1997) also found the supression of glioblastoma tumour growth in nude mouse xenografts by both a non-specific PS-ODN and a monoclonal antibody to the flk-1 receptor, the mechanism of attributed to the perturbance in natural ligand binding.

1.11 OLIGONUCLEOTIDES UNDER CLINICAL TRIALS

In vivo pharmacokinetic data have enabled some antisense ODN therapies to be developed to the stage of clinical trails (reviews by Szymokowski, 1996 and Akhtar *et al.* 1997). ODN modification, conjugation of moieties and development of delivery systems have overcome the problems of stability and uptake in some cases. Table 1.4 lists some ODNs currently under trial, highlighting the fact that an antisense-based therapy may become reality. Doses range between

Table 1.4: Some Oligonucleotides under Clinical Trials

Target	ODN	Method of Admin.	Trial Phase	Reference
p53 Protein mRNA	PS-ODN (OL1)p53	I.V.	Phase I	Bayever *et al.* (1993)
gag protein mRNA of HIV	PS-ODN (GEM 91)	I.V.	Phase I/II	Zhang *et al.* (1995)
Human Cytomegalo-Virus (CMV)	PS-ODN (ISIS 2922)	Intra-Vitreal	Phase III	Azad *et al.* (1995)
Intracellular Adhesion Molecule-1 (ICAM-1)	PS-ODN (ISIS 2302)	I.V.	Phase II	Henry *et al.* (1997)

As early as 1992, the clinical potential of an ODN was evaluated when a PS-ODN, called (OL1)p53, complementary to mRNA of the p53 protein, which is considered a tumour supressor, was systematically administered to a human patient with acute myelogenous leukemia (Bayever *et al.* 1992). Although the patients leukemia did not respond to treatment, no other significant toxicological affects were observed, be it neurological, cardiovascular, respiratory or renal. Interestingly, the *in vitro* growth of cells from the patient, removed following treatment, was inhibited (Bayever *et al.* 1992). This early study highlighted the potential of ODN therapy, currently (OL1)p53 is under phase I of clinical trial (Bayever *et al.* 1993). The differences between the *in*

vitro and *in vivo* reactions to ODNs is made evident by this study, highlighting the need for both types of study.

ISIS 2922, an ODN targetting CMV-induced restinosis (reviewed by Nokta *et al.* 1997), is the most advanced ODN in terms of clinical trials, phase III (Azad *et al.* 1995). It is not clear whether the effect of ISIS 2922 is by a true antisense mechanism, however trials continue due to favorable anti-viral activity and little associated toxicity. Recent evaluations of ISIS 2922 toxicity and efficacy by Flores-Aguilar *et al.* (1997) have revealed no toxicity to the retina of rabbits or gineau pigs *in vivo*. Human clinical trials of this ODN involving HIV patients suffering from retinitis are currently underway.

The anti-HIV ODN, GEM 91, also appears promising as a potential therapeutic agent, remaining intact for up to 6hrs in patient plasma (Zhang *et al.* 1995). Temsamani *et al.* (1997) in a more recent study found a similar plasma stability profile in HIV-infected individuals, 57% of GEM 91 remaining intact 30 minutes after a 3hr i.v. infusion period. The liver and kidney were found to have the highest ODN uptake, the brain the lowest degree of uptake although the amounts were not quantified with respect to the initial dose.

High costs of ODN production are being jointly addressed by pharmaceutical and biotechnology companies, the fatality of some disease states and great potential for use as an anti-viral, anti-cancer and anti-inflammatory agent indicates that ODNs will continue to be researched as viable therapeutic agents.

1.12 AIMS OF PROJECT

Antisense technology has now reached an exciting phase of development. Advances in the key issue of improving delivery *in vitro* and *in vivo* now serve as a catalyst for rapid progress to the development of an antisense based therapeutic agent and is the subject of this thesis.

Although there have been a multitude of studies investigating the enhancement of ODN delivery to cells *in vitro* and *in vivo*, there has not emerged one clear method which can be adapted to all cellular systems. Research has shown that ODN uptake differs from one system to another due to different ODN chemistry, the delivery method utilised and the model cell or animal system under investigation. Therefore it appears that to achieve ODN uptake there must be extensive independant study of the cell system, the delivery method designed to specifically cater for that system.

Macrophages are known to harbour many pathogens including HIV. Targetting these cells with anti-HIV ODNs have been under intense study since the development of antisense ODN technology. This project aimed to investigate several methods for enhancing *in vitro* ODN delivery to a macrophage cell line, with a future aim of developing a method for delivery into an animal model *in vivo*.

The macrophage surface mannose receptors were targetted with a novel mono-mannose ODN, designed and synthesised in the department, assessing its binding characteristics and mechanism of uptake. A novel approach was taken in that the mannose moiety was directly conjugated onto the ODN as opposed to the use of a linker group. The cellular association and uptake of these novel mannose conjugated ODNs was assessed in two different macropahge cell lines, and the binding characterisitics investigated in order to determine the efficiency of ODN delivery.

Three individual lipophilic ODN conjugates were also assessed for their binding and uptake characteristics in the same macrophage cell line. This approach aimed to determine whether increasing the lipophilicity of the ODN would enhance uptake, also investigating the mechanism by which cellular uptake occurs.

Lysosomes are a major intracellular site for ODNs internalised by RME or FPE. The intracellular stability of ODNs and ODN conjugates was evaluated in rat lysosomal extracts.

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1 CELL CULTURE

2.1.1 CELL LINES

2.1.1.1 U87-MG Human Glioblastoma Cells

A human Glioblastoma malignant glioma cell line, U87-MG, purchased from the European Cell Culture Collection (ECACC No. 89081402) was used. These cells are derived from a malignant glioma (Grade III) by the explant technique (Poten, *et al.*, 1968). U87-MG cells are an adherent cell line which grows as a monolayer in Dulbeccos-Modified Eagles Media (D-MEM) supplemented with 10% foetal calf serum (FCS), 1% glutamine and the antibiotic Gentamycin (0.1%).

2.1.1.2 J774 Murine Macrophages

The murine macrophage cell line J774 was a gift from Ciba, Horsham, UK. This cell line is adherent, having morphologic, adherent and phagocytic characteristics of macrophages (Ralph *et al.* 1975), growing as a monolayer in D-MEM supplemented with 10% FCS, 1% penicillin/streptomycin, 1% non-essential amino acids (NEAA) and 2% glutamine.

2.1.1.3 RAW264.7 Murine Macrophages

The macrophage cell line, RAW264.7, was a gift from Dr.R. Juliano, University of North Carolina, USA. These cells are derived from Abelson leukaemia virus induced lymphoma macrophages (Ralph *et al.* 1977; Raschke *et al.* 1978). This adherent cell

line grows as a monolayer in Dulbecco's-MEM supplemented with 10% foetal calf serum, 1% glutamine and 1% penicillin/streptomycin.

2.1.1.4 A431 Epidermal Carcinoma Cells

Epidermal carcinoma A431 cells (Ullrich *et al.* 1984), a gift from Dr.P.Nicklin (Ciba, Horsham, U.K.), were an adherent cell line maintained as a monolayer in Dulbecco's-MEM supplemented with 10% foetal calf serum, 2% glutamine and 1% penicillin/streptomycin.

2.1.2 MAINTENANCE OF CELL LINES

All cell lines were cultured in 75cm^2 tissue culture flasks (Sarstedt, Leicester, U.K.) incubated at 37°C with 5% CO_2. When cells reached confluency they were passaged by trypsinisation and diluted to a ratio of 1 to 3. Trypsinisation involved washing the cellular monolayer (75cm^2 tissue culture flask) with pre-warmed phosphate buffered saline (PBS) and incubation of the cells with 5ml of trypsin/EDTA (Sigma-Aldrich, Poole, U.K.) for 10 minutes at 37°C, or until the cells were seen to be detached by microscopy. The trypsin was neutralised and diluted by addition of fresh supplemented Dulbeccos-MEM (10ml), and the required volume transferred to new flasks containing 15ml D-MEM. All cell culture equipment was obtained from Gibco BRL (Paisley, Scotland, U.K). The number of times a cell line is trypsinised and diluted is referred to as the passage number. The U87-MG cells used were passage number 127, the J774 and RAW264.7 macrophage cells passage number 62 and 48 respectively.

2.1.3 MYCOPLASMA TESTING

Cultured cells were tested for contamination with mycoplasma using an adapted DNA fluorochrome technique (Flow Laboratories, Scotland, U.K.). The monolayer cells were grown in 8-well chamber slides (NUNC, Gibco BRL, Scotland) and used at 50 to 80% confluency. Post incubation the media was removed and the cells covered in fixative 2 (fixative 1:PBS in a 1:1 ratio) for 2 to 5 minutes, this was subsequently removed and replaced with fixative 1 (glacial acetic acid and methanol in a 1:3 ratio) for 10 minutes. Fixative 1 was removed and the cells allowed to air dry, after which they were submerged in stain solution (25mg bis-benzimide trihydrochloride, 5mg merthiolate, 50ml Hanks Balanced Salt Solution) (all reagents from Sigma-Aldrich, Poole, U.K.) for 10 to 30 minutes at room temperature. This stain was removed, the cells washed in double distilled water, ddH_2O, (3 x 0.5ml) allowing one minute soaking time between each wash and air-dried. The monolayer was covered in mounting solution (4.662g citric acid monohydrate, 8.247g di-sodium phosphate, 500ml glycerol made up to 1 litre with ddH_2O) and examined under a fluorescence microscope (Jenamed Fluorescence microscope) under oil immersion. Positive mycoplasma results are indicated by tiny random spots of fluorescence, 0.1-0.3μm in diameter, particularly surrounding the nucleus. All cells subsequently used were tested as mycoplasma free.

2.1.4 TRYPAN BLUE VIABILITY TEST

Cell viability was monitored prior and during each cellular assay using the following method. Detached cell suspensions (following trypsinisation) were mixed in 0.4% trypan blue solution (Sigma-Aldrich, Poole, U.K.) in a 1:1 ratio and a drop of the mixture placed on a haemocytometer (Fisons, Loughborough, U.K.). Cells were counted and number calculated by standard techniques, cells stained blue being non-viable cells. The percentage of viable cells was calculated, suspensions more than 5% non-viable being discarded.

2.1.5 COUNTING AND SEEDING CELLS

Cells were detached from flasks by trypsinisation (section 2.1.2) and seeded into wells or chambers at particular densities, a haemocytometer being used to determine cell number (section 2.1.4), appropriate dilutions being made by the addition of fresh Dulbeccos-MEM. For cellular association studies 24-well tissue culture plates (Falcon, Beckton-Dickinson, New Jersey, U.S.A) were used cells being seeded in quadruple wells for each assay. U87-MG cells were seeded at a density of 1 x 10^5 per ml per well and the macrophages (RAW264.7 and J774) seeded at 5 x 10^5 per ml per well in these 24-well plates unless otherwise stated.

Cells were seeded, 250µl of stated density per chamber, in chamber slides when required for photography in duplicate chambers, being seeded at a lower density than for the cellular association studies, U87-MG at a density of 5 x 10^4 per ml per well and the macrophage cell lines at 1 x 10^5 per ml per well. Cells were allowed to adhere to the chambers for 20 hours prior to experiments.

2.2 OLIGONUCLEOTIDE SYNTHESIS

2.2.1 THE CHEMISTRY OF OLIGONUCLEOTIDE SYNTHESIS

ODNs are referred to as 'PO' if a phosphodiester backbone, and 'PS' if a phosphorothioate. All unconjugated/unmodified PO and PS ODNs were synthesised using an automated DNA/RNA synthesiser (Applied Biosystems, Model 292, Warrington, U.K.) using phosphoramidite chemistry. All ODNs were synthesised on a 0.2µmol scale, the concentrations of each reagent is intrinsically programmed into the synthesiser program. All reagents for ODN synthesis were from Cruachem, Scotland, U.K.. Synthesis was initiated in a 3' to 5' direction from a nucleoside linked

by its 3'-hydroxyl group to a solid support (comprising of silica glass beads called Controlled Pore Glass, CPG), the next nucleoside added at the 5'-end.

The reaction steps involved in ODN synthesis, as performed by the Applied Biosystems DNA/RNA synthesiser, are summarised:-

2.2.1.1 Deprotection

The nucleoside attached to the CPG is protected at the 5'-end by a dimethoxytrityl (DMT) group (acid labile moiety) which is cleaved with trichloroacetic acid (deprotection) leaving a reactive 5'-hydroxyl group. The cleaved DMT group is subsequently flushed away from the column to prevent re-tritylation. An indirect means of monitoring the efficiency of each coupling step is by observing the colour of this flushed DMT, which is a bright orange coloured cation in an acid solution. The more intense the orange colour the greater the number of DMT groups removed from nucleosides on the CPG, therefore the greater the number of deprotected nucleosides for reaction in the next step.

2.2.1.2 Coupling

Phosphoramidites are chemically modified monomeric nucleosides, bearing specialised functional groups. To maintain stability when under storage a di-isopropylamino group on a 3'-trivalent phosphorus moiety is present to prevent side reactions. To aid in solubility a β-cyanoethyl group on the 3'-phosphorus moiety and protecting groups on the exocyclic amines of the bases 'A', 'C' and 'G' (removed after completion of ODN synthesis, section 2.2.1.6) and a DMT group as described in section 2.2.1.1. Following deprotection the column is made anhydrous (free from nucleophiles such as water) by washing with acetonitrile and drying with an argon reverse flush to remove residual acetonitrile. Next, phosphoramidite nucleosides are added to the column with tetrazole, a weak acid which brings about protonation of the nitrogen atom of the di-isopropylamine group on the 3'-phosphorus. This protonated

nitrogen now being susceptible to nucleophillic attack by the deprotected nucleoside on the CPG, the resulting bond being a phosphite triester (section 2.2.1.4). Excess tetrazole ensures that all the phosphoramidite is activated and excess phosphoramidite relative to free 5'-hydroxyls of the nucleoside on the CPG ensures over 98% coupling. This reaction is completed in about 30 seconds due to the reactivity of the phosphoramidite.

2.2.1.3 Capping

Not all the nucleosides on the CPG undergo phophoramidite coupling, an estimated 2% will fail to couple, and these unreacted nucleosides need to be blocked so as to prevent synthesis of short truncated ODNs. This step is termed 'capping', the unreacted 5'-hydroxyl group of the nucleosides is acetylated by an agent formed from acetic anhydride and N-methylimidazole which are simultaneously delivered to the column. Free 5'-hydroxyl groups react with the acetylating agent, being replaced with an acetyl group that is no longer able to take part in any further reactions, an argon reverse flush removing excess reagents.

2.2.1.4 Oxidation

The phosphite (trivalent phosphorus) triester inter-nucleotide linkage is very unstable, being susceptible to acid and base cleavage. Immediately following capping the phosphite triester is stabilised by oxidation that will convert it into a stable pentavalent phosphate triester (phosphotriester). Iodine, in a basic tetrahydrofuran (THF) solution with water, is a mild oxidant performing the oxidation step. An iodine-pyridine complex forms when the iodine-water-pyridine-THF mixture enters the column, this complex forming an adduct with the phosphite triester. This adduct is decomposed by water which donates an oxygen group resulting in the production of a pentavalent phosphotriester inter-nucleotide group. The column is once again flushed with argon and acetonitrile washes.

2.2.1.5 Phosphate Deprotection and Cleavage from the Support

Finally, after the complete ODN sequence has been synthesised it must be cleaved off the CPG, which is performed by four treatments with ammonium hydroxide cleaving the base-labile ester linkage between the CPG and the 3'-hydroxyl of the initial nucleoside. Simultaneously the ammonium hydroxide removes the β-cyanoethyl protecting groups on the phosphates (section 2.2.1.2). The resulting free ODN being collected in glass vials in an ammonium hydroxide solution.

2.2.1.6 Base Deprotection

This step is performed after the ODN is removed from the synthesiser. The ODN still needs further deprotection, the base protecting groups (section 2.2.1.2) have to be removed which is achieved by heating the ODN (in ammonium hydroxide solution, Sigma-Aldrich, Poole, U.K.) to 55°C for 8 to 15 hours. The ammonia acts as a nucleophile, which attacks the amide protecting groups.

2.2.1.7 Sulphurisation (Phosphorothioate Synthesis)

For the synthesis of phosphorothioates the oxidation step, section 2.2.1.4, is replaced by a sulphurisation step. The reagent used is tetra-ethylthiuram disulphide (TETD) in acetonitrile converts the phosphite group into a phosphorothioate triester at RT, taking 15 minutes, all other steps/reagents being the same as for phosphodiester synthesis.

2.2.2 PREPARATION OF UNCONJUGATED OLIGONUCLEOTIDES

All unconjugated/unmodified PO and PS ODNs were synthesised on an automated DNA/RNA synthesiser, using the chemistry summarised above. The sequences of the ODNs can be seen in the relevant chapters. Following deprotection, the ODNs were evaporated to dryness under vacuum (Savant, Leicestershire, U.K.) and stored at -

20°C. Before use ODNs were reconstituted in 200μl of ddH_2O. Aliquots of these stock solutions were made and stored at -20°C.

2.2.3 CALCULATION OF OLIGONUCLEOTIDE MOLECULAR WEIGHT AND CONCENTRATION

Optical density (OD) measurements were performed on spectrophotometer to allow calculation of ODN concentration. To do this the MW and the extinction coefficient (∈) at 260 nm wavelength were calculated for each ODN.

MW = [(249 x nA) + (240 x nT) + (265 x nG) + (225 x nC) + (64 x n-1)] +2

n base = number of bases of that type. n = total number of bases.

∈ (μmol) = (8.8 x nT) + (7.3 x nC) + (11.7 x nG) + (15.4 x nA)] x 0.9

Once the MW and ∈ have been calculated, OD values obtained can be converted into milligrams (mg) using the following equations:-

1 mg has an OD of ∈/MW

1mg = X OD units

1 OD unit = ^{1} / X mg

mg / MW = mmol

2.3 RADIOLABELLED OLIGONUCLEOTIDES

2.3.1 RADIOLABELLING PROTOCOLS

ODNs were radiolabelled with ^{32}P-ATP, either at the 3'-end, 5'-end or internally, as specified. Following radiolabelling, ODNs were separated from unlabelled strands and free radiolabel by polyacrylamide gel electrophoresis (PAGE), method described in section 2.3.2.

2.3.1.1 3'-End Labelling

The reaction used 100pmol ODN, [a^{32}P] di-deoxy-ATP (specific activity 650 Ci per mmol) (ICN Biomedicals Ltd, U.K.) and the 3'-end labelling reaction mixture Calf Thymus Terminal Transferase enzyme (1μl), the provided buffer (containing 1M potassium cacodylate, 125mM Tris-HCl, 1.25mg/ml Bovine serum albumin) and 2.5mM cobalt chloride solution (5μl) (all Boehringer Mannheim, East Sussex, U.K.). The reaction mixture was incubated for hr at 37°C, then purified by a Sephadex NAP-10 column (Pharmacia-Biotech, Upsala, Sweden) and the resulting solution evaporated to dryness (Savant, Leicestershire, U.K.). The NAP-10 column was used to remove potassium cacodylate and un-incorporated ^{32}P-ATP, which were found to distort ODN migration by PAGE. The dried ODNs were reconstituted in 50μl glycerol (50% containing 0.1% bromophenol blue and 0.1% xylene blue) and separated by native 20% PAGE. Labelled ODNs were purified from the gel according to the protocol in section 2.3.2.4.

2.3.1.2 5'-End Labelling

ODNs (100pmol) were 5'-end labelled with γ-dATP-[^{32}P] (ICN Biomedicals, Oxfordshire, U.K.) using the enzyme T4 Polynucleotide Kinase in ligation buffer (660mM tris-HCl pH7.6, 50mM $MgCl_2$ and 100mM DTT) and ATP solution (10mM

ATP in 50mM tris-HCl pH7.5) (all Bioline Ltd., London, U.K.), as instructed by the manufacturer, incubating the mixture for 30 minutes at 37°C. Labelled ODNs were purified from the gel according to the protocol in section 2.3.2.4.

2.3.1.3 Internal Labelling

Internal labelling was performed in two stages, firstly labelling the 5'-end (with ^{32}P) of one half of the ODN followed by ligation to the other half, at the labelled end, the ^{32}P label being incorporated internally. The ODN to be labelled internally was synthesised in two halves (termed primer 1 and 2, from the 5'- to 3'-end), *i.e.* a 20-mer sequence synthesised as two 10-mers and a complementary sequence (termed the template) were synthesised (standard synthesis method section 2.2.2). Using a protocol developed by Dr.N.Normund (personal communication, Aston University), primer 1 (10pmol), which had been previously 5'-end labelled and purified (section 2.3.1.2), was mixed with the molar equivalent of primer 2 (10pmol), and 10 molar equivalents of template ODN (100pmol) in 1µl ligation buffer (660mM tris-HCl pH7.6, 50mM $MgCl_2$ and 100mM DTT), 0.5µl ATP solution (10mM ATP in 50mM tris-HCl pH7.5) (both Bioline Ltd.) and 5µl NaCl solution (0.8M), heating the mixture for 5 minutes at 95°C, cooling to RT and incubating at 4°C for 3 hours. DNA Ligase enzyme (Bioline Ltd.) was subsequently added to make a final volume to 10µl, further incubating for 3 hours at RT, purifying the labelled and unlabelled fragments by PAGE (section 2.3.2).

2.3.2 POLY ACRYLAMIDE GEL EELECTROPHORESIS (PAGE)

2.3.2.1 Native and Denaturing Electrophoresis

Radiolabelled (either 3'-end, 5'-end or internal with ^{32}P) ODNs were separated by electrophoresis on 20% (w/v) polyacrylamide gels (29:1 acrylamide:bis-acrylamide, Severn Biotech, Worcester, U.K.), the ^{32}P-ATP label enabled visualisation of

separated ODN strands by autoradiography (section 2.3.2.3). Two types of PAGE were used depending on the application, either non-denaturing (native) or denaturing (7M urea). For purification of labelled ODNs from unlabelled strands and free radiolabel native PAGE was used (Sambrook *et al.* 1989). Labelled ODNs for cellular and stability experiments (section 2.4) were separated by denaturing PAGE, the high urea content serving to ensure ODNs were linear for accurate size determination.

2.3.2.2 Electrophoresis

Gel electrophoresis 20cm x 22cm glass plates (Bio-Rad, Hertfordshire, U.K.) were assembled as directed by the manufacturer, separated by spacers (1.5mm thickness). To 20% polyacrylamide (50ml of native or denaturing) 600µl of 10% ammonium persulphate (ICN Biomedicals, Oxfordshire, U.K.) and 40µl of N,N,N',N'-tetramethylethyl-diamine (TEMED, ICN Biomedicals, U.K.) were added and the gel was poured. A well forming comb was inserted between the plates and the gel allowed to polymerise for 30 minutes at room temperature. The comb was then removed and the wells washed in 1x tris-borate-EDTA buffer (TBE: 90mM tris base, 90mM boric acid and 2mM EDTA). The gel was placed in a Bio-Rad electrophoresis tank containing 1x TBE and the gel pre-run for 30 minutes at 25mA. Samples were loaded and electrophoresed at 30mA for 1.5 hours unless stated otherwise. During electrophoresis the approximate location of the migrating ODNs can be estimated relative to bromophenol blue (0.1% w/v) and xylene cyanol (0.1% w/v) dyes in the sample loading buffer, which electrophorese as 8-mer and 28-mer ODNs (Sambrook *et al.* 1989).

2.3.2.3 Autoradiography

Following electrophoresis, one of the glass plates was removed and the surface blotted to remove excess TBE. The gel was covered in Saranwrap and autoradiography film (Amersham, Buckinghamshire, U.K.) placed on the gel in an autoradiography cassette (Amersham, Buckinghamshire, U.K.). Native gels, containing newly labelled ODN, were exposed to film for between 1 to 2 minutes at room temperature and 12 hours to

several days exposure at -70°C for other gels containing less radioactive labelled ODN such as during a stability study (section 2.4). Exposed films were developed with standard developer and fixer solutions as recommended by the manufacturer (Fisons, Leicestershire, U.K.).

2.3.2.4 Gel Extraction of Oligonucleotides

Autoradiography of 3'-end, 5'-end and internal labelling samples after native PAGE (section 2.3.1.1, 2.3.1.2 and 2.3.1.3 respectively) revealed a band corresponding to the labelled ODN. This was then extracted from the gel, as ODNs are highly water soluble, this was achieved by a simple crushing and soaking method (Sambrook *et al.* 1989). Labelled ODN, as visualised by autoradiography, were pinpointed, sections of the gel were cut out through the film and crushed with a blunt ended Pasteur pipette and shaken in 2ml of ddH_2O for between 1 to 6 hours (the more concentrated the sample, the greater the radioactivity). Gel fragments were decanted and the ddH_2O, containing the labelled ODN, pipetted off into microfuge tubes, which were evaporated to dryness. The soaking, shaking and decanting step was repeated at least 3 times to extract the majority of labelled ODN. These labelled ODNs often contained tiny fragments of gel which were removed by a NAP-10 Sephadex column (Pharmacia-Biotech, Sweden) and stored at -20°C in a perspex box (Scotlab, Scotland, U.K.) until use.

2.3.3 SCINTILLATION COUNTING

Radiolabelled ODNs were assessed for radioactivity by scintillation counting (LSC 2500, Camberra-Packard Scintillation Analyser 1900, Berkshire, U.K.), according to the manufacturers instructions, using pre-set programs for ^{32}P or ^{3}H, for labelled ODNs and mannose/mannitol respectively. Samples were placed on 10ml scintillation cocktail (OptiPhase HiSafe 3, Wallac, Finland) in scintillation vials (Sarstedt,

Leicestershire, U.K.) and counted for 5 minutes each. A reference vial containing scintillation cocktail only was used to monitor background radiation levels, values higher than 30 counts per minute (CPM) being considered too high and recounted.

2.3.4 SCANNING DENSITOMETRY OF AUTORADIOGRAPHS

In order to quantify different ODNs, relative to one another as separated by PAGE, autoradiography (section 2.3.2.3) was performed and the dark bands representing labelled ODNs on the film analysed using scanning densitometry. Densities of each band relative to each other on the autoradiography film were represented as peaks by the program, the area under which enabled calculation of relative quantities. Densitometry enabled quantification of ODN degradation, intact ODN bands being compared to those for smaller fragments and free ^{32}P-label.

2.4 OLIGONUCLEOTIDE STABILITY PROFILES

Cellular stability studies were performed to monitor dissociation of ^{32}P-label from the ODN, as caused by nuclease action. Electrophoresis and autoradiography were used to assess labelled ODN, taken up by cells in culture, in comparison to a control labelled ODN of the same length which had not been exposed to cells. Apical medium (that the cells are bathed in) containing labelled ODN were collected after the stated incubation period/condition, taking care not to remove cells. These apical samples were electrophoresed on a 20% acrylamide/7M urea gel for 1.5hr at 30mA. Autoradiography was used to visualise separated labelled fragments (resolved in terms of size). Degradation would result in ODN fragmentation, autoradiography detecting labelled fragments of different lengths and free ^{32}P-ATP. The degradation pattern

being compared with the intact control ODN. Stability studies were performed parallel to an uptake experiment.

2.5 OLIGONUCLEOTIDE CELLULAR ASSOCIATION STUDIES

2.5.1 OPTIMISING THE STANDARD CELLULAR ASSOCIATION PROTOCOL

The protocol described in section 2.4.2 was used to determine the fraction of ODN cellularly associated under given conditions. Prior to these cellular association studies a standard protocol was established, to optimise the assay conditions. U87 cell monolayers were investigated, this data being used as standard for all cell lines. The number of washes required to remove unbound and loosely bound surface ODNs was investigated. Following incubation with labelled ODNs at 37°C, cells were washed in a volume of 0.5ml six consecutive times with pre-warmed phosphate buffered saline/0.05% azide (PBS-azide, Sigma-Aldrich, U.K.). Each fraction was placed in separate vials containing 10ml of scintillation cocktail, and the radioactivity assessed by scintillation counting.

2.5.2 STANDARD CELLULAR ASSOCIATION PROTOCOL

Uptake studies were performed on semi-confluent monolayers of cells (seeded into quadruple wells) as follows. Cells were seeded at different densities depending on the cell line (U87-MG seeded at a density of 1 x 10^5 cells/ml, J774 and RAW264.7 seeded at of 5 x 10^5 cells/ml) into sterile 24 well tissue culture plates, and allowed to attach for approximately 20 hours. Following incubation the serum containing media

was removed, cells being washed twice with PBS (warmed to 37°C) to remove traces of serum and treated with ^{32}P-labelled ODNs in 200µl of serum free Dulbeccos-MEM, incubating at the stated temperature and incubation period (ensuring the monolayer was not disturbed during pipetting). 0.1µM of unlabelled ODN spiked with labelled ODN (approximately 100,000cpm) was used for all experiments unless otherwise stated. After incubation the apical Dulbeccos-MEM on the cells was removed and pooled together with PBS-azide washes (4 x 0.5 ml), taking care not to disturb the monolayer, placing in vials containing 10ml scintillation fluid. The cellular monolayers were removed from the wells by solubilising with 3% Triton-X100 (Sigma-Aldrich, U.K.) treatment (3 x 0.5ml) and placed in separate scintillation fluid vials, the radioactivity of all vials being assessed by scintillation counting (section 2.3.3) using the appropriate radioisotope programme.

Cellular association values of the ODNs were calculated as the percentage radioactivity, either per well of replicate sterile 24 well tissue culture plates or per cell number, defined in each experiment.

2.5.2.1 Oligonucleotide Efflux from Cells

Cells not only internalise exogenous compounds but also exude them, a balance between endocytosis and exocytosis existing in order to maintain the cellular volume. Besterman *et al.* (1981) discovered the phenomenon of exocytosis (efflux) in their studies of the fluid phase marker sucrose whose accumulation appeared to decrease with increasing time in alveolar macrophages and lung fibroblasts. Investigations by these researchers showed intact non-metabolised sucrose being exocytosed out of the cell, the process being necessary to maintain cell volume homeostasis. Similar experiments by other researchers have been reviewed by Besterman *et al.* (1981), other fluid phase markers being shown to have similar endocytic and exocytic processes. Therefore it may be possible that such an equilibrium exists in other cell types

The mechanisms by which such efflux occurs is unclear, although Besterman *et al.* (1981) suggest the involvement of intracellular compartments which exocytose the their contents. Many other researchers have investigated the process by which ODNs are effluxed out of cells (Gao *et al.* 1993; Temsamani *et al.* 1994; Tonkinson *et al.* 1994), the process being dependent on both the cell line and nature of the ODN.

Tonkinson *et al.* (1994) describes a two-compartment model for the exocytosis of ODNs from cells. A 'shallow' compartment into which PO-ODNs localise and rapidly efflux out of, and a 'deep' compartment into which PS-ODNs localise and efflux out of at a slower rate is proposed (Tonkinson *et al.* 1994).

The amount to labelled ODN that effluxes out of the cell were investigated. The standard cell association protocol was followed (section 2.5.2) (incubating for 1 hour at 37°C) after which the apical Dulbeccos-MEM medium was removed and placed in a scintillation vial along with PBS washes (3 x 0.5ml), fresh serum free Dulbeccos-MEM medium was added to the cells, incubating for a further 30 minutes at 37°C. Following incubation the apical media was again removed and placed in separate scintillation vials along with the PBS washes, this was repeated at defined time intervals (60, 90, 180 and 300 minutes after the initial 1 hour incubation), apical media and PBS washes for each interval being placed in separate scintillation vials. Finally the cells were solubilised as in the standard cell association protocol (section 2.5.2), again being placed in separate vials and radioactivity assessed by scintillation counting (section 2.3.3).

2.5.2.2 Cellular Association of Oligonucleotides and Fluid Phase Markers

Comparisons of cellular association of the test ODN to cells were made to a fluid phase endocytosis marker i.e. a molecule known to enter cells by fluid phase endocytosis. Many studies investigating the binding and uptake characteristics of ODNs utilise fluid phase markers. Besterman *et al.* (1981) and Beltinger *et al.* (1995)

describe the use of sucrose, Stein *et al.* (1993) used albumin and Nakai *et al.* (1996) used inulin as markers of fluid phase endocytosis, the latter two studies showing a significantly lower level of fluid phase marker association to the cells compared to an ODN.

The cellular association of labelled ODNs and a fluid phase marker (either ^{3}H-mannose or ^{3}H-mannitol) over a series of incubation periods was evaluated (30, 60, 90, 180 and 300 minutes), the standard cell association protocol (section 2.5.2) being followed after each incubation period. Cells were plated (section 2.1.5) in quadruple wells for each ODN or fluid phase marker and for each incubation period, radioactivity being assessed by scintillation counting using the appropriate program (section 2.3.3).

2.5.2.3 Effect of Temperature

Cellular associations of ODNs to cells in culture were assessed during incubation temperatures of 4°C and 37°C. At the physiological temperature of 37°C many enzymes will function at their optimum, laws of thermodynamics stating that energy is required for reacting molecules to interact with one another. Proteins, lipids and other molecules also generally require the temperature to be at 37°C in order to maintain their native conformations.

A decrease in temperature to 4°C would therefore slow down the functioning of all active, energy requiring processes, ATP production (an enzymatically controlled process) therefore ceasing. If a process was dependent on cellular energy then it should be significantly inhibited at 4°C, such a result being an early indication of energy dependency.

To assess the effects of temperature on the cellular association of ODNs, cells were incubated at 37°C (incubator) and 4°C (fridge) for the stated incubation period, after which the standard cell association protocol, section 2.5.2, was followed.

2.5.2.4 Effect of Metabolic Inhibitors

Metabolic inhibitors were used to further investigate the mechanism employed by the ODNs to associate with cells. Two of the most widely used metabolic inhibitors, used in combination for ODN investigations are sodium azide, NaN_3, (Loke *et al.* 1989) and 2-deoxyglucose, 2DG, (Wu Pong *et al.* 1994a).

NaN_3 is an inhibitor of the enzyme cytochrome oxidase, which is an essential respiratory enzyme complex found in the inner mitochondrial membrane. Cytochrome oxidase is crucial in the transport of electrons from NADH to oxygen, a process that leads to the liberation of hydrogen ions (H^+). A build up of H^+ (protons) leads to the formation of an electrochemical gradient across the mitochondrial membrane, these protons subsequently flow down this gradient through an enzyme, ATP synthase, which converts the energy released into that required to form the bond between ADP and P_i resulting in ATP formation.

2-Deoxyglucose if a glycolytic inhibitor. Glycolysis is the process by which glucose is catabolised, a process involving nine steps resulting in the generation of two net ATP molecules from ADP and P_i. As with the respiratory process involving cytochrome oxidase, glycolysis is crucial for ATP, energy, production, providing the cell with energy for normal active processes.

Cells seeded in 24-well plates were pre-incubated in Dulbecco-MEM either with the two inhibitors (10mM NaN_3 and 50mM 2-DG) or without for 30 minutes at 37°C after which 10μl of ODN solution (made up of labelled and unlabelled ODN to 0.1μM) was added, the plate being swirled for 1 to 2 minutes to ensure the ODN

mixed in with the Dulbeccos-MEM, the plate incubated for a further for 60 minutes at 37°C, after which the standard cell association protocol (section 2.5.2) was followed.

2.5.2.5 Effect of pH

The effect of different pH on the cellular association of ODNs was investigated in order to gain information about the degree of protein association. Proteins can be made more cationic by a decrease of exogenous pH, their attraction to the anionic ODNs increasing, leading to an ionic interaction. A number of researchers (Goodarzi *et al.* 1991; Kitajima *et al.* 1992; Beck *et al.* 1996; Hawley *et al.* 1996) have used pH change to investigate ODN-protein interactions, a decrease in pH increasing this interaction.

Dulbeccos-MEM (pH approximately 7.3) was replaced with HBSS. HBSS (9.8g per 1L sterile double distilled water containing 0.01% w/v phenol red and 5mM D-glucose) was buffered with either HEPES (to produce a pH of either 7.0 or 8.0) or MES (for pH 5.0 or 6.0) (all reagents from Sigma-Aldrich, U.K.). The standard cell association protocol (section 2.5.2) was followed, pre-warmed serum free buffered HBSS containing the ODNs incubated with the cells for 60 minutes at 37°C.

2.5.2.6 Effect of the Proteolytic Enzyme Pronase

Pronase is a commercially available proteolytic enzyme mixture produced from the micro-organism *Streptomyces griseus* isolated by Narahashi *et al.* (1967). Isolation was originally from the culture broth of this microorganism, subsequent studies into the action of Pronase revealing it to be composed of a mixture of several proteinases and peptidases (including endopeptidases and exopeptidases). The exact number of these enzymes in the mixture being unknown. Due to Pronases mixed proteolytic enzyme composition it has a very broad specificity for proteins. This broad specificity makes Pronase equally efficient for assessing surface protein association to trypsin,

which has often been used for the same purpose (Wu Pong *et al.* 1992, 1994a and 1994b).

The standard cell association protocol was followed as described in section 2.5.2 up to the step after the PBS-azide washes (4 x 0.5ml). Prior to solubilisation of the cells a solution of 0.25% w/v Pronase in HBSS (0.5ml) was added to each well and incubated for 15 minutes at 4°C after which this Pronase solution was removed along with PBS-azide washes (3 x 0.5ml), being placed in separate scintillation vials. The standard 3% Triton-X100 solubilisation step was then performed, all vials being assessed by scintillation counting (section 2.3.3).

2.5.2.7 Effect of Self or Cross Competition

Self and cross competition experiments were performed in order to determine the specificity of ODN and conjugate binding to the cell surface. The cellular binding capacity of cells can potentially be limited by binding sites on the membrane surface, if a specific receptor is responsible for binding a ligand then saturation can occur. Self competition experiments, involving addition of cold unlabelled ODN to cells incubated with a standard amount of labelled ODN, allows the receptor theory to be assessed. If cellular association of the labelled fraction is depleted by addition of the unlabelled ODN then competition is probably occurring, and the cell may only have a limited number of ligand binding sites, which would become saturated with unlabelled ODN.

Cross competition enables the degree of specificity for binding to the cell surface to be assessed. A depletion of cellular association in the presence of non-self ODN would indicate that the same sites were being bound, *i.e.* competition, therefore the binding molecule on the cell surface not being very specific.

Cellular association of ODNs in the presence of excess unlabelled ODN (either the same *i.e.* self, or different *i.e.* non-self) were investigated, to assess whether competition for binding would occur. The standard quantity of labelled and unlabelled ODN (section 2.5.2) was added to cells and cellular association assessed, this being the control (section 2.5.2). The test condition of labelled and unlabelled ODN supplemented with the stated quantity of unlabelled ODN (either self or non-self according to experiment), investigated in parallel to the control, incubating for 60 minutes at 37°C. The standard cell association protocol was followed (section 2.5.2).

2.5.2.8 Effect of Competition with Polyanions

Polyanionic molecules, negatively charged species, are able to bond with cellular proteins via ionic interactions, certain sites within the protein structure having a positive charge. ODNs are polyanionic molecules, to assess whether ODNs and conjugates were binding to RAW264.7 cells via ionic interactions their cellular association was competed with excess non-nucleic acid polyanions, dextran sulphate (Wu Pong *et al.* 1992; Kajio *et al.* 1992; Fell *et al.* 1997) and heparin (Bennett *et al.* 1985; Ishai-Michaeli *et al.* 1992; Fell *et al.* 1997). The fact that these polyanions are non-nucleic acid molecules enables the specificity to be determined in terms of ionic interaction, determining whether the cell will bind any type of molecule on the basis of its charge as opposed to other properties.

As with cross and self-competition experiments, polyanion competition can determine the specificity of surface binding and also determine whether the interaction between the ODN and surface binding molecule is of an ionic nature.

The effects of competition in the presence of an excess of unlabelled non-nucleic acid based polyanion was investigated, either dextran sulphate (M.W. 10 000, cell culture grade, Sigma-Aldrich, U.K.), or heparin (sodium salt, cell culture grade, Sigma-Aldrich, U.K.). Cells were pre-incubated with the polyanion in serum free Dulbeccos-

MEM for 15 minutes at 37°C after which this media was aspirated off, the cells washed in PBS (3 x 0.5ml) and the standard cell association protocol followed (labelled ODN added, incubating for 60 minutes at 37°C, section 2.5.2).

2.5.2.9 Effect of Monensin

Ligand/receptor complexes once internalised by RME are found in intracellular acidic compartments, called lysosomes, as discussed in section 5.1.1. The dissociation of these complexes within the cell is thought to involve the acidic pH within the lysosomes. Monensin is an antibiotic from the micro-organism *Streptomyces cinnamonensis*. The structure of monensin is such that the oxygen atoms are orientated in the centre where they are available for complexation to sodium ions. Therefore they surround alkyl groups and render the molecule lipid soluble and enable diffusion through the membrane.

Monensin is a proton/sodium (Na^+/H^+) ionophore (Mollenhauer *et al.* 1990) and is frequently used as an agent to investigate RME. Monensin is a monovalent ether antibiotic, alkyl groups cover its surface making it lipid soluble and therefore able to diffuse through the plasma membrane, and has a 10-fold higher affintiy for Na^+ than K^+ (Mollenhauer *et al.* 1990). These studies determine whether the mechanism by which a ligand is trafficked in the cell involves acidic compartments (Wileman *et al.* 1984, Wu Pong *et al.* 1994a and 1994b; Tonkinson *et al.* 1994, Nakai *et al.* 1996). Monensin mediates the exchange of Na^+/H^+, causing an irreversible collapse of the H^+ gradient that exists across the lysosome membrane, from the interior to exterior cytoplasm. Once the gradient across the acidic compartments is collapsed by monensin the pH within these compartments increases. Processes that are dependent on low-pH, such as ligand/receptor dissociation, are therefore prevented (Wileman *et al.* 1985; Mollenhauer *et al.* 1990), thereby blocking ligand release into the cytoplasm, preventing transfer to other compartments and subsequently preventing receptor recycling back to the plasma membrane. In effect, both endocytosis and exocytosis processes are inhibited.

Monensin is stated as not affecting the internalisation mechanism directly, the uptake and binding of particles not being affected. However, intracellular trafficking is affected, Mollenhauer *et al.* (1990) review some if its actions; preventing the trafficking of molecules by the golgi, inhibiting late processing events within the golgi (terminal glycosylation, proteolytic cleavage), impeding transfer of molecules to secondary lysosomes and disrupting the recycling of membrane fragments back to the cell surface, transfer being more affected than processing events. Monensin does not appear to exert any change to the rate of exerts protein synthesis and ATP levels, having an effect at very small concentrations (0.01 to 1.0μM).

To investigate the effects of the endocytosis disrupting agent, monensin (Sigma-Aldrich, U.K.), cells were pre-incubated in 200μl serum free Dulbeccos-MEM containing monensin (10μM per well) and 5% dimethyl sulphoxide (DMSO, Sigma-Aldrich, U.K.), which was required to aid monensin solubility (a sonicator being used to ensure complete dissolution), for 30 minutes at 37°C, the control being cells in 200μl serum free Dulbeccos-MEM/5% DMSO. After pre-incubation ODN solution was added in a volume of 10μl, the plate being swirled to mix the ODN with the Dulbeccos-MEM, this was followed by further incubation for 300 minutes at 37°C after which the standard cell association protocol was followed (section 2.5.2).

2.5.2.10 Effect of Phenyl Arsine Oxide and Chloroquine

Phenly arsine oxide (PAO) and chloroquine, like monensin, are used in investigating the involvement of RME in ODN cellular association. Chloroquine is a lysosomatropic agent, having a similar affect to monensin and neutralising intracellular acidic compartments, thereby preventing acidic pH dependant receptor/ligand dissociation (Wibo *et al.* 1974; Wu Pong *et al.* 1994a). Other functions of chloroquine have been reported as prevention of optimal lysosomal enzyme action and inhibition of endosome-lysosome fusion (Wu Pong *et al.* 1994a). PAO is used as an inhibitor of epidermal growth factor (Wiley *et al.* 1983; Knutson *et al.* 1992) and insulin RME, amongst other proteins (Hertel *et al.* 1985). The

mechanism of action is unknown, PAO being described as a trivalent arsenical, a molecule that can form bonds with sulphydryl groups on cell membrane surface molecules.

The protocol for assessing the effects of 100μM PAO and 100μM Chloroquine (both inhibitors of endocytosis) was similar to that described in section 2.5.2.9, pre-incubation times differing, cells being pre-incubated in serum free Dulbeccos-MEM containing PAO (containing 5% DMSO to aid PAO dissolution) for 5 minutes at 37°C, chloroquine pre-incubation being for 30 minutes at 37°C, after which the labelled ODN was added, the cells incubated for 300 minutes at 37°C and the standard cell association protocol followed (section 2.5.2). Control cells for the PAO experiment were incubated in serum free Dulbeccos-MEM containing 5% DMSO and serum free Dulbeccos-MEM (without DMSO) for the chloroquine controls.

2.6 STATISTICAL EVALUATION OF DATA

Data was statistically evaluated by a one-tailed student's 't' test, using the Excel 5.0 computer program. The confidence level (p) used was 0.05.

2.7 CELL PHOTOGRAPHY

In order to assess the binding of fluorescent species to cells, RAW264.7 and J774 macrophages were seeded (250μl of 1 x 10^5 cells per ml) into the chambers of an 8-chamber tissue culture slide (NUNC, Gibco BRL, Scotland) and incubated in normal serum containing Dulbeccos-MEM overnight at 37°C (5% CO_2). The apical media was removed from each chamber, cells washed with pre-warmed (37°C) PBS (2 x

0.5ml) and 200µl of serum free D-MEM (pre-warmed) containing the fluorescent species added. Cells were incubated with the fluorescent species for hr at 37°C (the chamber slide wrapped in aluminium foil) after which the apical D-MEM was removed, cells washed in PBS (2 x 0.5ml) and the walls of the chamber removed as directed by the manufacturer. After air drying, a coverslip was dropped onto the cells that were subsequently visualised under an inverted Janamed fluorescence microscope using a green filter, at the stated magnification.

CHAPTER 3: A NOVEL MONO-MANNOSE CONJUGATE ODN

3.1 INTRODUCTION

There are major problems associated with developing antisense ODNs into a viable, efficient alternative to conventional drug therapy. Theoretically ODNs have the potential to combat many disease states at the molecular level, by having highly specific genetic targets. Overcoming the problems of stability, cellular uptake and intracellular trafficking are issues, which must be addressed before ODNs take a place in everyday drug therapy.

Macrophages are a good target for antisense ODNs, being both a natural harbour for the Human Immunodeficiency Virus (HIV) and other viruses and express a number of different membrane receptors which can potentially be exploited for ODN delivery. This chapter describes a potential mechanism to enhance ODN delivery into the cultured murine macrophage cell line RAW264.7 by targetting their plasma membrane surface mannose receptor. A single mannose moiety was conjugated to an antisense ODN targetting the Nitric Oxide Synthase mRNA (section 3.2.2). Previous approaches to conjugating mannose groups to ODNs have involved the use of large, bulky linker or carrier molecules (Bonfils *et al.* 1992a and 1992b). The approach to mannose conjugation described here directly links this sugar to an ODN, eliminating the bulky linker/carrier component, which may cause an antigenic response or confer steric hindrance to binding the mannose receptor.

3.1.1 MEMBRANE LECTINS

Membrane lectins are a type of cell surface receptor that recognise and bind ligands bearing carbohydrate moieties. Cell-cell interaction involves various signalling molecules. These can be small peptides composed of only a few amino acid residues such as Enkephalin, which is involved in the inhibition of pain pathways; fatty acid derivatives such as Prostaglandin E_2 , which causes constriction of smooth muscle; steroids like Cortisol which triggers the metabolism of proteins, carbohydrates and lipids; or large molecules such as Insulin, a protein involved in carbohydrate metabolism. Some signaling proteins, such as hormones, possess a carbohydrate moiety and are termed glycoproteins. Follicle Stimulating Hormone is a glycoprotein, which stimulates ovarian follicles, as is Leutinising Hormone which stimulates oocyte maturation (Alberts *et al.* 1989).

Cell surface glycoproteins that bind carbohydrate moieties are commonly known as membrane 'lectins', functioning in a similar manner to receptors but being distinctive in that their ligands contain carbohydrate moieties. The first studies on membrane lectins centered on plants. Concanavalin A is a commonly used, commercially available lectin from black beans (Palomino, 1994). The information gained from plant lectins was subsequently used for the elucidation and characterisation of animal lectins. Table 3.1 lists some animal lectins and their functions. Membrane lectins are capable of binding both endogenous ligands (Stahl *et al.* 1978; Schleslinger *et al.* 1980; Shepherd *et al* .1981; Otter *et al.* 1991) and exogenous or foreign glycoprotein ligands (Sharon *et al.* 1989). They are able to distinguish their ligands from the vast array of complex structures, which exist extracellularly. Since the discovery of lectins there have been a multitude of studies characterising their precise role and function, and research is being directed towards their exploitation for drug delivery, this has recently been reviewed by Palomino (1994).

Table 3.1: Studies Targetting Cell Surface Receptor using Specific Ligands Conjugates.

Receptor	Ligand	Cell System	Result	Reference
Mannose	β-glucoronidase or mannose-BSA	Rat Alveolar Macrophages	Mannose receptor recycles	Tietze *et al.* (1982)
Asialo-glycoprotein	Asialo-glycoprotein	Rats (*in vivo* infusion)	Compartments for uncoupling receptor /ligand complexes	Geuze *et al.* (1983)
Mannose	Mannosylated ferritin	Chicken bone marrow macrophages	Endocytic receptor function involving coated pits	Rossi *et al.* (1984)
Mannose	β-glucoronidase	Rabbit Alveolar Macrophages	Presence of receptor /ligand in lysosomes	Wileman *et al.* (1985)
Mannose	Mannose neo-glycoprotein	Rabbit and rat macrophages	Endocytic process and receptors re-used	Wileman *et al.* (1986)

Extensive information about lectins has enabled their categorisation into a number of different types according to localisation, binding properties and structure (Drickamer *et al.* 1988 and 1993). Lectins are categorised into C-type and S-types referring to Ca^{2+}-dependant and thiol-dependant mechanisms, respectively. In some lectins only a portion of the structure is responsible for ligand binding, termed the carbohydrate recognition domain (CRD), whereas other portions can have functions such as membrane anchorage and possess collagenous sequences (Kim *et al.* 1992). Study of the amino acid composition of C-type lectins show a high degree of conservation within the CRD (Drickamer *et al.* 1993). Other

properties shared by the C-type lectins are the extracellular location, and varied carbohydrate ligand specificity and differences in solubility (Drickamer *et al.* 1988).

S-type lectins may be either extracellular or intracellular, are buffer soluble, and β-galactosides are their main ligands (Drickamer *et al.* 1988). C-type lectins are found in serum and the extracellular matrix, many being membrane bound, where they mediate glycoprotein endocytosis. Further classifications can be made within the C-type category, based on the location of the lectin, the type of ligand bound and processed (endogenous or exogenous) and function (Drickamer *et al.* 1993).

In 1974, Ashwell and Morrell characterised the first membrane lectin on the surface of hepatocytes. They found this lectin to have an affinity for D-galactose and *N*-acetyl-galactosamine only, and it was named the asiaylo-glycoprotein-lectin. Serum glycoproteins bearing galactose residues are bound (by this hepatic lectin), internalised and transported to lysosomes where they are degraded (Falasca *et al.* 1992). Many membrane lectins have since been (Drickamer *et al.* 1993, Palamino *et al.* 1994). The endothelial cells of the hepatic system (Tolleshaug *et al.* 1984) and macrophages bear mannose receptors (Stahl, 1992) which bind glycoproteins bearing mannose and fucose residues.

Monsigny *et al.* (1988) reviews the membrane lectins found on tumour, lymphoid and myeloid cells. It is suggested that glycoprotein sugar moieties expressed on the membrane alter as the cell undergoes malignant transformation (Warren *et al.* 1978; Lotan *et al.* 1985; Meromsky *et al.* 1986). This would therefore alter the glycoprotein recognition processes of receptors. Alternatively the receptor protein itself may alter, affecting ligand recognition, affinity, activity or a combination of these functions.

Metastasis is the distant spread of a malignant tumour from its site of origin, via the bloodstream, the lymphatic system or body cavities (Alberts *et al.* 1989). Metastasis is a complex process that is poorly understood, which is proposed to

involve ligand recognition, binding and internalisation. Membrane lectins may be involved in the metastasis process. With the knowledge that cell surface glycoproteins mediate cell migration, any change in sugar composition, due to malignant transformation, may lead to the development of metastatic properties (Meromsky *et al.* 1986; Dennis *et al.* 1987; Dube, 1987). Malignant cells expressing aberrant lectins are proposed to aggregate with each other, with other tumour cells or with non-malignant host cells, therefore spreading (Sharon *et al.* 1989). The mechanism, by which this alteration in carbohydrate composition occurs, is (as yet) unclear. It has been hypothesised that as oncogenes are involved in transformation and differentiation processes (Ohlsson *et al.* 1987), they may also bring about alterations in the expression of the enzymes that control biosynthesis of glycoprotein carbohydrate moieties.

The β-galactose lectin, found on B16-melanoma cells, is hypothesised to be involved in tumour metastasis (Meromsky *et al.* 1986; Lotan *et al.* 1985). Lotan *et al.* (1985), and later Meromsky *et al.* (1986), used a monoclonal antibody (mAb 5D7) against the galactose lectin to investigate cell aggregation and colony formation. Lotan *et al.* (1985) demonstrated dose dependent reduction (up to 100%) in colony formation of various tumour cell lines, including B16-melanoma cells, in the presence of the anti-galactose lectin-mAb 5D7. Meromsky *et al.* (1986) studied the effect of asialofetuin, a glycoprotein bearing galactosyl residues and a ligand of the galactose lectin on B16-melanoma tumour cells. Tumour cells were found to aggregate crosslinking between cells mediated via this ligand. The antibody, mAb 5D7, was found to reduce tumour cell aggregation *in vivo* and therefore lung colonisation by 90% and *in vitro by* 80%. It was proposed that binding of this antibody to the galactose lectin blocked interaction between adjacent cells. However total inhibition of aggregation was not achieved in the presence of the antibody, probably due to the existence of other cell surface molecules involved in metastasis and cellular interaction.

The lectin of one cell type can potentially bind to a lectin-receptor or cell surface carbohydrate-containing molecule of another cell resulting in aggregation, either or both being a tumour cell causing metastasis. If the metastatic potential is related to

membrane lectin activity then understanding these recognition and binding systems may be crucial to defining and combating the metastatic state.

Targetting a receptor of a particular cell type with the purpose of enhancing delivery of an ODN as opposed to blocking function of that receptor has a number of advantages. Discrimination can be made between cell types, *i.e.* between normal and abnormal cells (Monsigny *et al.* 1988), or different cells within a homogeneous mixture in tissues or fluids. Therefore a high degree of specificity can be achieved because certain cell surface molecules of particular cells can be targetted.

3.1.2 MEMBRANE SURFACE GLYCOPROTEINS

Nature has exploited proteins and nucleic acids for the function of carrying a biological code. It is now believed that carbohydrates perform a similar function, having the capacity to build structures of differing sequence and numbers of monomeric units in the same manner as proteins and nucleic acids. However, they have an added variation to the types of structures they can form, this stems from their capacity to link to one another in different positions and anomeric configurations (α or β linkages) (Sharon *et al.* 1989; Drickamer *et al.,* 1993). Therefore, the fact that sugar units can be assembled in a vast array of geometric and stereochemical forms, raises the potential for many structures conveying recognition signals (Drickamer *et al.,* 1988; Palomino, 1994). Many biological functions, including cell recognition, migration and the immune response, utilise carbohydrates as an integral part of their systems. Membrane lectins play an integral role in this recognition system (Sharon *et al.* 1989). Glycoproteins are widespread in living organisms, fulfilling vital functions as enzymes, toxins etc.

Both prokaryotic and eukaryotic cells, with the exception of mycoplasmas, bear glycoproteins on their cell surface. These molecules have varying functions from enzymatic activity, cell-cell adhesion and recognition to structural support (Drickamer *et al.* 1988). Prokaryotes like bacteria have a complex network of

fibers, which constitute the bacterial cell wall, comprising of peptidoglycans (polysaccharides cross-linked to polypeptide chains), lipo-polysaccharides, lipoproteins, and porins (channel forming proteins) amongst others. Some bacteria have an additional layer surrounding the cell wall, for example pathogenic bacteria such as *Clostridium.* This is often termed the *s* -layer and comprises of glycoproteins, bearing protruding sugar residues.

All eukaryotic cells bear sugar moieties on their cell surface, particularly those of the reticuloendothelial system (the liver, spleen and kidney). In higher vertebrate cells the Major Histocompatability Complex (MHC) is composed of glycoproteins. The MHC binds foreign antigens and presents them to lymphocytes for destruction. As it became clear that nearly all cells express glycoproteins on their surface, it also became apparent that these carbohydrate moieties conveyed a biological code (for receptor binding and signalling), determined by the number and configuration of these protruding residues (Sharon *et al.* 1989).

3.1.3 MACROPHAGES AND MEMBRANE LECTINS

Macrophages play an essential role in the immune response, *which* leads to the destruction and elimination of foreign molecules. They have a vast array of functions including the scavenging and phagocytosis of cell debris and invading foreign matter (Sharon, 1984; Monsigny *et al.* 1994). They can also act as an antigen presenting cell *i.e.* stimulating T-lymphocytes (Alberts *et al.* 1989) and secrete cytotoxic chemicals (Alberts *et al.* 1989, Oda *et al.* 1988, Lefkowitz *et al.* 1991). A whole series of events occur in response to host attack by a foreign molecule. Intricate cascades of events arise during this response involving a great number of different cell types, which interact with one another. Central to these interactions are surface membrane receptors. It has been reported that macrophages express at least 30 different receptors on their surface giving rise to the potential for over 30 different ligands to bind to these cells making them ideal for the study of receptor targetting.

A number of membrane lectins are found on the surface of macrophages, amongst them the mannose-6-phosphate lectin, the galactose/N-acetylglucosamine lectin, and the mannose lectin (Stahl *et al.* 1984, Shepherd *et al.* 1990, Stahl *et al.* 1992). These receptors play an essential role in macrophage function, the galactose lectin is proposed to be involved in distinguishing tumour cells from normal cells and destroying these tumour cells (Sharon *et al.* 1989).

The mannose receptor, found on the macrophages of rats, mice and humans (Kery *et al.* 1992), mediates a process known as lectino-phagacytosis whereby microorganisms bearing mannose and fucose residues are bound and phagocytosed (Sharon *et al.* 1989). The configurations of residues are not commonly found at the termini of glycoproteins on the surface of mammalian cells or on serum glycoproteins, they are however common on microbial cells such as bacteria (Felipe *et al.* 1991), fungi (Warr 1980) and parasitic organisms (Wilson *et al.* 1986; Ezekowitz *et al.* 1990; Ezekowitz *et al.* 1991). The plant toxin, ricin, is also a mannosylated glycoprotein, which has been shown to be cleared by liver sinusoidal cells (Magnusson *et al.* 1993). Macrophages also have a scavenging function whereby endogenous self ligands, bearing residues in a configuration that is recognised, are bound, internalised and eliminated from the system, auch as lysosomal enzymes which are glycoproteins.

Felipe *et al.* (1991) describe the peritoneal macrophage phagocytosis of the bacterium *Escherichia Coli*.. A major soluble constituent of yeast cell walls is mannan, which is mannose rich and is orientated such that it is extracellularly exposed. Mannan is often used as a competitor for putative mannose receptor ligands. Mannan and anti-mannose receptor antibodies inhibited *Escherichia Coli* phagocytosis, with neither glucose or galactose blocking any effect. This behaviour is consistent with the bacterium binding to the mannose receptor of macrophages, in a similar manner to parasitic binding, prior to phagocytosis.

The mannose receptor was shown to be involved in fungal cell binding by Warr in 1980. Murine lung macrophages were found to bind the yeast strain *Candida*

krusei via their mannose rich glycoprotein coat, binding being both temperature and Ca^{2+} dependent (C-type), and subject to competition with natural ligands for the receptor such as mannose, glucosamine or other mannose rich glycoproteins such as β-glucoronidase or Horse Radish Peroxidase. No effect on binding was seen in the presence of mannitol or galactose.

The parasite *Leishmania donovani* has a carbohydrate rich surface. Investigations by Wilson *et al.* (1986) on binding of this microorganism to monocytes and monocyte-derived macrophages revealed characteristics consistent with binding to the mannose receptor. As with other ligands of the mannose receptor, *Leishmania donovani* binding to monocyte-derived macrophages was significantly reduced in the presence of mannan and inhibited by the neo-glycoproteins mannose-BSA and fucose-BSA. In freshly isolated monocytes, which characteristically do not have mannose receptors, there was no inhibition of *Leishmania donovani* binding in the presence of mannan, in comparison with a 59.9% inhibition in monocyte-derived macrophages, leading to the conclusion that this parasite used the mannose receptor as a means of host invasion by receptor-mediated phagocytosis by macrophages. The intracellular fate of this parasite was investigated using ammonium chloride, a weak base that increases the intra-vesicular pH of lysosomes. Such disruption would prevent the dissociation of the receptor-ligand complex in lysosomes, if this were the mechanism employed in receptor recycling. Treatment with ammonium chloride showed a reduction in mannan binding and parasite binding as the intra-vesicular pH was increased indicating that both ligands are internalised and the receptor recycled by a similar mechanism. A more recent study by Ezekowitz *et al.* (1991) showed association between the parasite *Pneumocystis Carini,* which also bears a mannose rich glycoprotein surface, and mannose receptors on both human and rat alveolar macrophages.

The scavenging function of the mannose receptor involves the binding and internalisation of endogenous ligands (Stahl *et al.* 1978; Schlesinger *et al.* 1980; Shepherd *et al.* 1982; Otter *et al.* 1991). Tissue plasminogen activator (TPA), a glycoprotein bearing mannose rich oligosaccharides on its outer surface, is a proteinase released into the bloodstream by vascular endothelial cells to degrade

fibrin structures within blood clots. It has been suggested that the mannose receptor play a key role in clearance of this mannose rich enzyme from the bloodstream. Otter *et al.* (1991) studied the binding of TPA by bovine alveolar macrophages. This study describes mannose receptor binding characteristics for TPA which are similar to known ligands of the receptor, such as decreased binding at acidic pH or in the presence of mannose-albumin, mannose and fucose monosaccharides; galactose-albumin, galactose and N-acetyl-galactosamine were less potent inhibitors. De-glycosylation of TPA, *i.e.* removal of mannose oligosaccharide moieties, lead to a decrease in affinity of this ligand for the mannose receptor confirming that glycosylated TPA can function as a natural ligand for the mannose lectin.

Lysosomal glycosidases, such as α-hexosaminidase and β-glucoronidase, which are potentially harmful when released under aberrant conditions, are also thought to bind mannose receptors. Such recognition and internalisation is used as a mode of clearance from the plasma (Stahl *et al.* 1980). Schlesinger *et al.* (1980) showed the clearance of mannose-terminated lysosomal glycosidases by the mannose lectin on alveolar macrophages *in vitro.* In contrast, when galactose terminated glycoproteins were used as a control these were not cleared by these macrophages.

The envelope protein of HIV is a glycoprotein, gp120, with a high mannose content. This protein is involved in one of the first steps in HIV infection of a host cell (Lifson *et al.* 1986; Curtis *et al.* 1992). Lifson *et al.* (1986) found Concanavalin A, a mannose binding lectin from lentils, inhibited *in vitro* infection of HIV virions, inhibition reduced when competed with α-methyl-D-mannoside. The researchers proposed that Concanavalin A interacted with envelope glycoprotein at the cell surface preventing infection via a mannose specific-lectin mediated mechanism. Curtis *et al.* (1992) investigated the binding of this glycoprotein to placental tissue, which does not possess the known cellular HIV receptor CD4, revealing gp120 to bind a C-type mannose binding protein, which had a higher affinity than CD4 for gp120 binding. These findings suggest that this mannose binding protein could potentially be used as a competitive agent to inhibit HIV binding to CD4 positive cells. Macrophages are known to be a harbour for

HIV, a major target for this virus. If the mannose receptor were involved in infectivity in some way then there exists the potential for competing this action by blocking this receptor.

The importance of the mannose receptor is therefore highlighted; possible role in cancer metastasis, a scavenging function and possible involvement in HIV infection. Therefore an ODN targetted to the mannose receptor can potentially have a number of functions depending on its target. As macrophages possess the mannose receptor, and are essential in the immune response as well as having a scavenging role, they were considered ideal as an *in vitro* cell culture model for evaluating an ODN delivery system based on targetting the mannose receptor.

The Murine macrophage cell lines RAW264.7 and J774 were used to assess association of mono-mannose-conjugated ODNs. The use of established cell lines ensures that a homogeneous cell population is studied. Both these Murine macrophage lines are derived from tumours, RAW264.7 cells from Abelson Leukemia Virus Induced BALB/c-Lymphocytic Lymphoma, and J774 cells from BALB/c tumour.

3.1.4 THE MACROPHAGE MANNOSE RECEPTOR

The mannose receptor is a 170-175kDa protein (Wileman *et al.,* 1983; Largent *et al.* 1984; Stephenson *et al.* 1987; Kuhlman *et al.* 1989; Kery *et al.,* 1992) which binds and internalises glycoproteins bearing mannose and fucose residues. It also binds N-acetylglucosamine and glucose but with a lower affinity (Lennartz *et al.,* 1987; Taylor *et al.* 1990; Pimpaneau *et al.,* 1991).

Extensive work has been performed on this lectin to elucidate its structure and function. The expression of the mannose receptor is tightly regulated. Primary macrophages, freshly isolated from lung or liver, express a significant number of these receptors. Monocytes, one of the three main types of white blood cell, do not

express this receptor until they leave the circulatory system and mature into macrophages (Roche *et al.* 1985). Bone marrow cells also only express the receptor when stimulated by colony stimulating factor (CSF-1). A number of macrophage cell lines do not express significant levels of the receptor *in vitro*, for example J774, P338.D1 and U937 (Stahl *et al.* 1982; Diment *et al.* 1987; Blum *et al.* 1991) but these may stimulated to increase their mannose receptor number. Lymphokines can down-regulate receptor expression (Imber *et al.* 1982), whereas non-inflammatory steroids stimulate expression (Shepherd *et al.* 1985). Diment *et al.* (1987) up-regulated mannose receptor expression in J774 cells by treatment with the drug 5-Azacytidine, a compound which irreversibly induces expression of repressed genes

3.1.5 STRUCTURE OF THE MANNOSE RECEPTOR

Various mannose-conjugated ligands have been used as probes to detect the presence of, and isolate, the mannose receptor. FITC labelled mannose-BSA conjugates and latex mini beads derivatised with mannose-BSA (Kataoka *et al.* 1985) have been used in addition to ^{125}I-labelled mannose-BSA (Stahl *et al.* 1980, Kataoka *et al.* 1985, Wileman *et al.* 1985, Lennartz *et al.* 1987). Blum *et al.* (1991) generated a specific antibody to the mannose receptor (using J774 mouse macrophages) to investigate receptor function. This antibody was found to react with both mouse and human mannose receptors. Homology has been identified between bovine (Otter *et al.* 1991) rabbit (Lennartz *et al.* 1987), rat (Wileman *et al.* 1986), mouse (Blum *et al.* 1991) and human (Stephenson *et al.* 1987; Kim *et al.* 1992) mannose receptor protein, which appears to be highly conserved between species.

The mannose receptor is a C-type lectin located in the plasma membrane, binding exogenous and endogenous ligands. The structure comprises of three domains, only one of which is associated with ligand recognition and binding. The first domain is a cysteine rich NH_2 terminal whose function is unknown, as is the

function of the Fibronectin type II domain, although it is hypothesised that this latter domain may play a part in promoting macrophage adhesion and spreading to tissue sites (Ezekowitz *et al.* 1990), and is involved in binding some bacteria which possess fibronectin receptors (Stahl, 1992). The domain, which binds ligands, is unique to the mannose receptor. It comprises eight segments, which are related to C-type CRDs, all of these branch from one single polypeptide chain (Ezekowitz *et al.* 1990; Taylor *et al.* 1992; Kim *et al.* 1992; Drickamer *et al.* 1993).

Extensive studies by Ezekowitz *et al.* (1990) and Taylor *et al.* (1992 and 1993) have characterised the structure of the mannose receptor. Different combinations of the eight CRDs have been cloned and expressed in fibroblasts *in vitro*, in order to elucidate the precise functions of each component (Taylor *et al.* 1992 and 1993). There are also two other domains in the mannose receptor, the N-terminal, cysteine rich and the fibronectin type II repeat domains, not found to be involved in ligand endocytosis. CRDs 1-3 were found to have only a weak affinity for ligands, with the majority of binding activity residing in CRDs 4-8. Only CRD4 was shown to bind small monomeric or dimeric ligands in the absence of the other domains. High affinity binding and endocytosis of a multivalent glycoprotein was shown to require CRDs 4, 5 and 7. It has been hypothesised that CRDs 5 and 7 directly interact with the ligand, aiding in tight binding to the receptor, however the receptor was most active and had the highest ligand binding capacity when all eight CRDs were fully functioning.

Ezekowitz *et al.* (1990) transfected mannose receptor cDNA into cells *in vitro*, control cells being transfected with the complementary DNA (cDNA) of an unrelated protein (phosphoglycerate kinase). Cells transfected with mannose receptor cDNA were able to bind and internalise the yeast *Candida albicans*, which was quantified, by a yeast staining method. Binding or ingestion of yeast was not detected in cells transfected with the phosphoglycerate kinase cDNA. Kery *et al.* (1992) performed binding affinity studies on purified human placental mannose receptors using oligosaccharides ligands of varying size and configuration. These studies showed that a ligand bearing only one sugar residue had a very low affinity for the receptor, and that affinity increased proportionally

with the number of residues present. These mannose oligosaccharides were isolated from mannan, a protein free natural ligand of the mannose receptor, demonstrating that the protein component of mannosylated-glycoproteins plays no part in mannose receptor binding.

As the mannose receptor has multiple CRDs, and ligands bearing multiple sugar residues have a high binding affinity for this receptor, it is probable that this receptor is flexible enough to bind a wide range of ligands.

3.1.6 MACROPHAGES AND RECEPTOR MEDIATED ENDOCYTOSIS

The process of RME was described in section 1.8.1. The exploitation of RME for drug delivery into cells has attracted much attention, leading to a large number of different receptors being targetted. Some studies of membrane lectins are summarised in Table 3.1.

RME has been studied extensively for the asialo-glycoprotein receptor and its galactosylated ligands (Geuze *et al.* 1983; Kato *et al.* 1996). Geuze *et al.* (1983) followed the fate of the internalised ligand/receptor complex in liver parenchymal cells. The receptor/ligand complex was internalised at clathrin coated regions of the plasma membrane, vesicles forming within the cell. Other intracellular vesicles had little receptor associated with them. Geuze *et al.* (1983) named these ligand containing vesicles "compartment for uncoupling of receptor and ligand" (CURL), and proposed that they transform into lysosomes where degradation of the ligand occurs. Receptors were also found in structures, resembling tubular extensions, containing very little ligand.

Rossi *et al.* (1984) investigated, via electron microscopy, the endocytosis of mannosylated ferritin by chicken macrophages. As in other studies, binding of the mannosylated ligand by the mannose receptor was reduced by competing ligands

such as mannan. The electron micrographs revealed a high density of mannose receptor-ligand complex in coated pit regions of the plasma membrane, which were invaginating. This data suggests that the mannose receptor follows this classic mechanism of receptor internalisation.

In 1985 Wileman *et al.* investigated the binding of a mannose rich glycoprotein, ^{125}I-β-glucoronidase to rabbit alveolar macrophages. After incubation of the cells with the ligand to allow binding and internalisation, lysosomes were fractionated from the plasma membrane by a percoll gradient method. This study revealed a concentration dependent, saturable binding of the mannose lacto-peroxidase ligand to the receptor, which was inhibited in the presence of mannan.

A multitude of reports has characterised mannose or mannosylated conjugate binding and internalisation by macrophages. Stahl *et al.* (1984) reviews the mechanism by which the mannose receptor is thought to function on alveolar macrophages. The receptor is thought to be recycled, however most receptors are sequestered in an intracellular pool, with only one fifth of the total being found on the extracellular surface at any one time. Stahl *et al.* (1980) found that treating cells with trypsin at 4°C destroyed mannose receptors, which subsequently lead to a 30% reduction in mannosylated ligand binding. The 70% of ligand that still bound were therefore accounted for by recycling from the intracellular pool of receptors to the cell surface. From the number of receptors per cell and the number of ligand molecules binding per minute per cell, the mannose receptor is calculated to recycle every 11 minutes. Receptor-ligand complexes on the cell surface accumulate and internalise at coated pits and vesicles. Once inside the cell the vesicle containing the complex is acidified via a proton pump and this acidic environment is thought to cause receptor/ligand dissociation. The receptor is returned to the cell surface for another cycle of RME, and the ligand transported to lysosomes.

Evidence for recycling of the mannose receptor was provided in a study by Tietze *et al.* (1982) who demonstrated that when the pH of intracellular compartments

was raised, by chloroquine and ammonium chloride, receptor/ligand dissociation was inhibited, preventing recycling of free receptor back to the cell surface for further ligand binding.

Monensin, a proton/sodium ionophore, which mediates the exchange of H^+ for Na^+ leading to an increase in pH (Wilcox *et al.* 1982), has been found to block receptor/ligand dissociation. Intravesicular acidity has been proposed to cause ligand release from its receptor in some systems. Wileman *et al.* (1984) investigated the effects of monensin on the internalisation of β-glucoronidase. Monensin blocked the dissociation of β-glucoronidase from the mannose receptor with no effect on binding of the ligand at the cell surface, thereby significantly slowing down uptake. This study also shows that ligand transfer from endosomes to lysosomes is blocked.

The conjugation of an ODN to mannose residues will potentially allow internalisation of this ODN along with the mannose-receptor complex, which occurs via RME (Lennartz *et al.* 1987; Basu *et al.* 1991; Kery *et al.* 1992). Many studies have used mannose conjugated to BSA (Stahl *et al.* 1982; Rossi *et al.* 1984; Wileman *et al.* 1985; Lennartz *et al.* 1987) or neo-glycoproteins (Wileman *et al.* 1986) to follow mannose receptor binding and endocytosis in macrophages (Stahl *et al.* 1982; Rossi *et al.* 1984; Wileman *et al.* 1985; Lennartz *et al.* 1987) or neo-glycoproteins (Wileman *et al.* 1986).

3.1.7 MACROPHAGE MANNOSE RECEPTOR TARGETTING BY MANNOSYLATED LIGANDS

In an early study Stahl *et al.* (1978) concluded that a cell surface receptor existed on rat alveolar macrophages, which bound glycoproteins and neo-glycoproteins (synthetic glyco-conjugates) bearing mannose, glucose or N-acetylglucosamine residues. Binding of the ligands mannose-BSA, N-acetylglucosamine-BSA and

glucose-BSA was found to be temperature dependent, being negligible at 4°C and at its most efficient at 37°C. Ligand binding was totally abolished by mannan, indicating competition and saturation was occurring as ligand concentration increased. Both competition and saturation data point towards a limited binding capacity, *i.e.* a limited number of binding sites, which is characteristic of a ligand/receptor binding system. Various mannans composed of sugar residues linked by bonds of differing configuration were used to study the binding preferences of the receptor. Stahl *et al.* (1978) found that the mannose receptor preferentially bound certain types of linkages, the carbohydrate moiety of the glycoprotein/glycoconjugate dictating the receptor affinity for a ligand. A later study by Kery *et al.* (1992) also investigated the effects of various mannose-oligosaccharides, with differing linkages, on binding to the human placental mannose receptor. Again, as with the Stahl *et al.* (1978) study, the finding was that oligomers with certain linkages bound more efficiently than others to the receptor.

Delivery of the enzyme, superoxide dismutase (SOD) to cultured mouse peritoneal macrophages has been achieved using mannosylated enzyme (Fujita *et al.* 1992; Takukura *et al.* 1994). SOD enzymatically degrades the superoxide anion, which is important for host defence and inflammation produced in macrophages by the oxygen metabolism cascade. Takukura *et al.* (1994) studied mannosylated-SOD binding and uptake and compared it to an non-mannosylated-SOD conjugate and galactosylated-SOD. Uptake of the mannosylated-SOD was significantly higher than the controls, as was the inhibition of superoxide anion release from the macrophages. Uptake of mannosylated-SOD (man-SOD) was significantly reduced at 4°C, in the presence of excess mannan or mannose and in the presence of the endocytosis inhibitor Cholchicine. Therefore it was concluded that the mechanism of uptake of the man-SOD into macrophages required energy, was competable by the presence of excess mannose residues and involved an endocytic mechanism, indicating that the mannose receptor mediates binding and internalisation of the mannosylated-conjugate. This study again highlights the potential of the mannose receptor for internalising ligands artificially conjugated to mannose residues, which may be utilised for the delivery of ODNs.

3.1.8 OLIGONUCLEOTIDE-CONJUGATES AS LIGANDS FOR THE MANNOSE RECEPTOR

Antisense ODNs conjugated to specific sugar residues have been delivered to cells *in vitro* by targetting their membrane lectins. Such targetting has proved successful in some cases, an enhancement in binding of these conjugates being seen compared to the unconjugated species. Deshpande *et al.* (1996) describe a successful system for ODN delivery by conjugation to the Epidermal Growth Factor (EGF), targetting the EGF-receptor of adenocarcinoma cells. ODNs have been delivered to hepatocytes via the galactose (asialo-) receptor (Wu *et al.* 1992; Lu *et al.* 1993). Parenchymal liver cells express the asialo-receptor on their surface, which is highly selective for galactose terminated glycoproteins, internalisation occurring via RME. Wu *et al.* (1992) targetted the asialo-receptor of hepatocytes with an ODN 21-mer antisense to the human hepatitis B virus.

Similar studies have also been performed for ODN targetting to the macrophage mannose receptor, as summarised in table 3.2.

Bonfils *et al.* (1992a and 1992b) targetted the mannose receptor of macrophage cell lines, Mahato *et al.* (1997) targetted rat macrophages *in vivo*, and Rojanasakul *et al.* (1997) targetted primary alveolar macrophages. Bonfils *et al.* (1992a) coupled a fluorescently labelled ODN to mannosylated BSA, this conjugate shown to bind and internalise to a higher degree than a non-mannosylated-BSA-ODN conjugate. Confocal microscopy showed a higher fluorescent intensity within intracellular vesicles for the mannosylated-BSA-ODN conjugate, whereas the non-mannosylated-BSA-ODN conjugate accumulated around the cell periphery. One of the pathways followed by ligands internalised by RME is sequestration into the acidic intravesicular compartments, endosomes and lysosomes, which are approximately pH5.0. Treatment of the cells with monensin, which neutralises acidic compartments (Mollenhauer *et al.* 1990) resulting in the release of their contents into the cytoplasm, after initial ODN conjugate treatment, lead to an

increase in fluorescent intensity within the cell cytoplasm, providing evidence for the initial intravesicular localisation of the ligand.

Table 3.2: Mannose Receptor Targetting by Mannosylated Ligands.

Ligand Complex	Cell System	Result	Reference
ODN Mannosylated BSA complex	J774 Macrophage cell line	Intra-vesicular localisation of ligand/receptor	Bonfils *et al.* (1992a)
Mannosylated strepavidin biotin ODN complex	J774 Macrophage cell line	Increased binding of mannosylated ligand	Bonfils *et al.* (1992b)
Mannosylated PLL ODN complex	Rat alveolar macrophages	17-fold increase in ODN binding, by a RME process	Liang *et al.* (1996)
Mannosylated PLL ODN complex	Rats dosed *in vivo* (by the tail vein)	Increased liver and spleen accumulation	Mahato *et al.* (1997)
Mannosylated PLL ODN complex	Rat alveolar macrophages	Antisense inhibition of ODN target (mRNA)	Rojanasakul *et al.* (1997)

Bonfils *et al.* (1992b) labelled an ODN with fluorescein at the 5'-end and biotin at the 3'-end. Strepavidin substituted with mannose residues was conjugated to the biotin-oligonulceotide-BSA complex. As in their previous study, Bonfils *et al.* (1992b) found a significantly higher degree of binding and internalisation of the mannosylated ODN conjugate compared with the non-mannosylated control in J774 cells, monensin revealed intravesicular localisation of the mannosylated conjugate within the macrophages.

More recently Liang *et al.* (1996) approached mannose receptor targetting of macrophages with poly-lysine (PLL) substituted with mannose residues and this complex conjugated to an ODN, finding a 17 fold higher degree of internalisation of the mannosylated-PLL-ODN conjugate compared to a non-mannosylated conjugate. Again the conjugate was found to be localised within endocytic vesicles. The ligand, mannosylated-PLL ODN conjugate, was however cytotoxic due to the PLL moiety. Mahato *et al.* (1997) and Rojanasakul *et al.* (1997) also used mannosylated PLL ODN conjugates to target the mannose receptor.

3.1.9 AIMS - A MANNOSE PHOSPHORAMIDITE DERIVATIVE

The above studies illustrate the potential to enhance uptake of mannosylated species into macrophages via mannose-receptor mediated endocytosis. The conjugates used in the above studies are however large and bulky species due to the BSA component (Liang *et al.* 1996: Mahato *et al.* 1997: Rojanasakul *et al.* 1997). Such large conjugates may affect the process of RME, reducing efficiency. The protein component of the conjugate can potentially bind to other proteins on the cell surface or the extracellular fluid, decreasing bioavailability. It could also sterically hinder binding of the mannose residues to the receptor, or have an immunogenic effect. Therefore it is desirable to reduce the size of conjugates by eliminating the protein component, by making the ODN-mannose link more direct.

Although the concept of developing carbohydrate based drugs and targetting membrane lectins should theoretically enable efficient targetting, many practical problems exist. Membrane lectins will only bind specific ligands, which have particular sugar orientations and are linked by specific bonds. Therefore conjugating drugs to these ligands and maintaining the integrity of the carbohydrate moiety and its binding to the receptor is a complex task.

Our approach, for ODN targetting to the mannose receptor, was to link the mannose moiety in a more direct manner, eliminating the protein component. The synthesis of a unique mannose phosphoramidite derivative was devised (Akhtar *et al.* 1995). This mannose phosphoramidite was used in the same way as a conventional nucleotide phosphoramidite, coupling being performed by an automated DNA synthesiser. The mannose phosphoramidite derivative was such that a 5'-end mono-mannose conjugated ODN was synthesised. This is a very novel approach to mannose conjugation to ODNs, a six-carbon linker being the only other component of the system. This linker was designed such that it carried the mannose group at one end and a hydroxyl group at the other, the hydroxyl group being converted into the phosphoramidite moiety.

Whether elimination of the protein component of mannose ODN conjugates would enhance mannose receptor recognition, binding and internalisation was investigated in this project. It is well known that ligands bearing multiple mannose residues are required by the mannose receptor for efficient binding. The novelty of such a conjugate necessitates preliminary studies, however, investigating viability of a mono-mannose phosphoramidite in terms of coupling to ODNs, stability and cellular association.

3.2 MATERIALS AND METHODS

3.2.1 MANNOSE PHOSPHORAMIDITE SYNTHESIS

Both the monomeric and dimeric mannopyranoside phosphoramidites were synthesised using commercially available methyl-α-D-mannopyranoside (Akhtar *et al.* 1995), the syntheses being performed by the Chemistry Department at Aston University. A simplified reaction scheme is outlined in figure 3.1.

The monomeric mannopyranoside phosphoramidite was used for ODN conjugation and subsequent cellular association studies for this project. The first step in the synthesis involves the protection of the non-reacting hydroxyl groups on the methyl-α-D-mannopyranoside, followed by the production of a chloro-α-D-mannopyranoside derivative (figure 3.1). Dimer synthesis requires the reaction between the methyl-α-D-mannopyranoside and the chloro-α-D-mannopyranoside derivative. This chloro group of the monomer or the dimer is then substituted by a protected 6-benzyloxy-1-hexanol group, a six carbon linker which will act as the spacer between the mannose residue and the ODN. The protecting group of the linker is subsequently removed and the hydroxyl group substituted with a phosphoramidite moiety. The resulting α-D-mannopyranoside phosphoramidite can be placed directly on the DNA/RNA synthesiser.

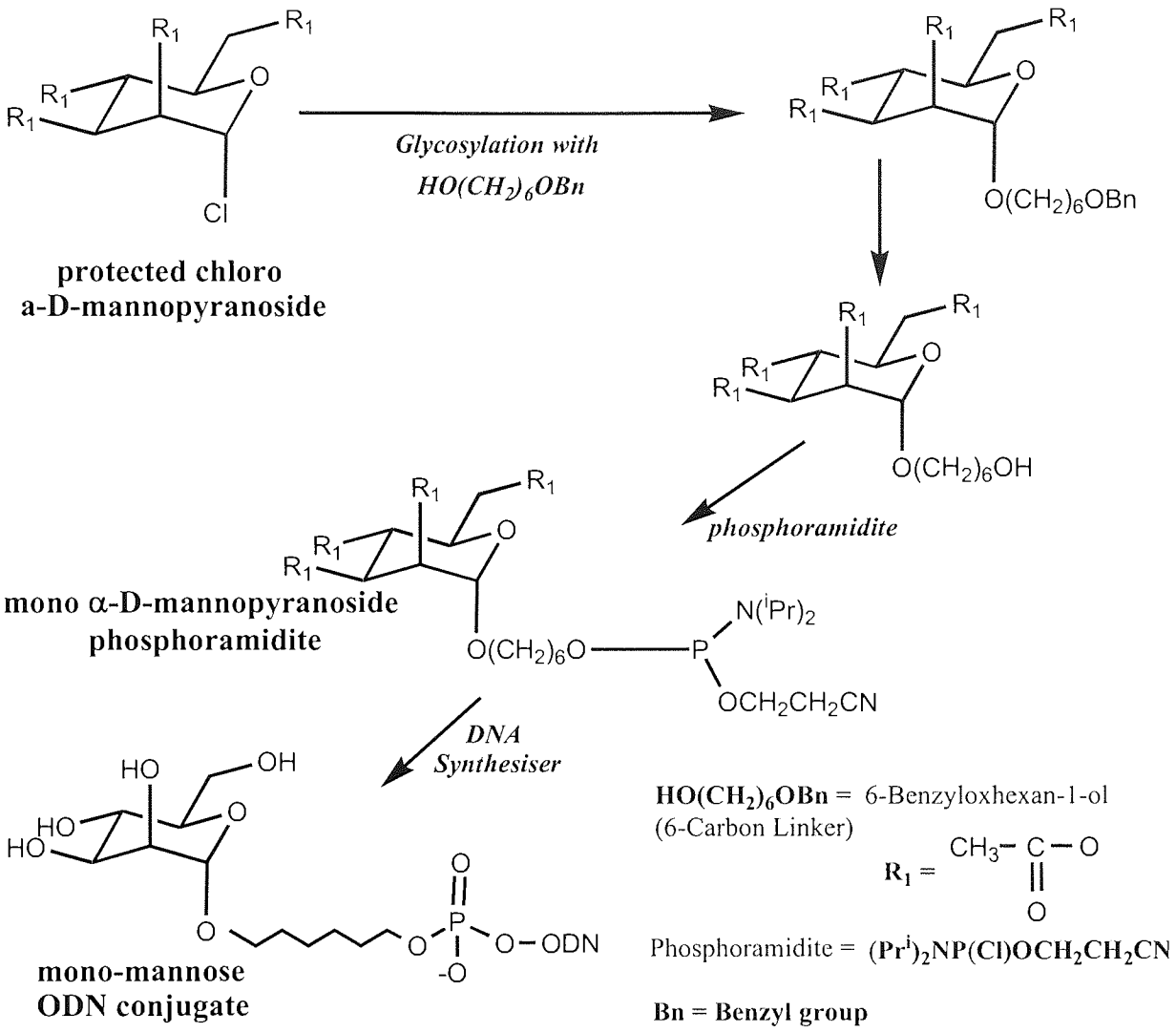


Figure 3.1: Schematic Representation of the Synthesis of Mono-Mannose Pyranoside Phosphoramidite. Non-reacting hydroxyl groups of the methyl-α -D-mannopyranoside are protected by acetyl groups, a 6-carbon linker group (benzyl alcohol) reacted with the non-protected chloride group and the hydroxyl group of the benzyl alcohol substituted with a phosphoramidite. The resulting mono-mannose phosphoramidite is subsequently placed on a DNA synthesiser for conjugation to an ODN.

3.2.2 OLIGONUCLEOTIDE SYNTHESIS

All unconjugated ODNs were synthesised and prepared for use according to the method described in section 2.2.2. All the ODNs used in this chapter were phoshodiester chemistry. Phosphorothioates were not synthesised due to the instability of the α-D-mannopyranoside phosphoramidite species. As outlined in section 2.2.1, phosphorthioate synthesis involves a longer reaction time than phosphodiesters, which would contribute to the degradation of the α-D-mannopyranoside phosphoramidite species.

3.2.2.1 Unconjugated Oligonucleotides

The ODNs used in the mono-mannose conjugate work were antisense to the Nitric Oxide Synthase (NOS) gene. Both conjugated and unconjugated ODNs were 15 bases long (15-mer). A 16 base and 12 base NOS sequence were also used, as part of characterisation of the mono-mannose conjugate, in the electrophoretic gel shift assay. The sequences of the NOS ODNs are displayed below: -

15mer NOS antisense sequence $^{5'}$ CCA GGG GCA AGC CAT $^{3'}$

15mer NOS sense sequence $^{5'}$ ATG GCT TGC CCC TGG $^{3'}$

16mer NOS antisense sequence $^{5'}$ G CCA GGG GCA AGC CAT $^{3'}$

12mer NOS antisense sequence $^{5'}$ GGG GCA AGC CAT $^{3'}$

3.2.2.2 Mono-Mannose Conjugated Oligonucleotide Synthesis

Synthesis of the mannose phosphoramidite is described above in section 3.2.1. This phosphoramidite species is very unstable once synthesised, the phosphorus group exists in a trivalent state (+3) which is extremely acid labile, and degrades rapidly in the presence of water. Therefore it is crucial that this unstable derivative is conjugated onto the ODN as soon as possible, where the trivalent phosphorus converts into a stable pentavalent state (+5). Due to this instability, only the PO-ODN and not the more biologically stable PS-ODN was synthesised, the latter chemistry requiring a longer time period for formation of the phosphorothioate linkage.

This mannose phosphoramidite derivative was placed immediately onto the DNA/RNA synthesiser, in a position for spare base bottles, which was programmed to add this phosphoramidite as the last base of the 15-D NOS sequence. Also synthesised was a conjugate made up of only a single base (the T-base of a column) and the mannose moiety, this 1mer-T conjugate was synthesised so that conjugation could be assessed by mass spectrometric analysis.

Resulting ODNs were collected in a different manner from the synthesiser compared to unconjugated ODNs. As described in section 2.2.1, the synthesiser dispenses ODNs in ammonia solution into sterile glass vials in, which are then deprotected. The synthesiser was programmed not to wash the newly synthesised mono-mannose ODN out of the column into vials as normally done. Therefore the ODN conjugate, after addition of the mannose posphoramidite to the 5'-end, remained in the column. This column was cut manually to release the CPG resin and the oligonuleotide conjugate. As for unconjugated ODNs, a deprotection step is needed, which was performed by shaking in ammonia-methanol overnight at room temperature. After deprotection the ODN was separated from the CPG resin by filtering through a 0.2μm filter, washing this filter out with water to remove any traces of remaining

ODN. The resulting mono-mannose conjugate was treated in the same way as unconjugated ODNs, vacuum drying, and reconstitution in 200µl of sterile double distilled water and storage in aliquots of smaller volumes.

The ODN sequence for the conjugate was the same as for the 15-D NOS ODN, the mannose group being bound at the 5'-end of the molecule: -

Mono-mannose 15mer NOS antisense sequence ('**X**' = Mannose moiety)

$^{5'}$ **X** CCA GGG GCA AGC CAT $^{3'}$

3.2.3 MASS SPECTROMETRIC ANALYSIS

The 1mer-T conjugate, synthesis described in section 3.2.2.2, was analysed by mass spectometry as a means of verifying conjugation of the mannose group onto the nucleotide. The method used was electrospray ionisation mass spectometry, VG platform II, as kindly performed by Dr. Paul Nicklin (Norvartis, U.K.). A peak at 624, the molecular weight of the species, was expected.

3.2.4 GEL SHIFT ASSAY

The 15D, 16D and mono-mannose conjugate NOS ODNs were 3'-end labelled and purified according to the method described in section 2.3.1.1. Purified 3'-^{32}P-end labelled ODNs were loaded separately by a 20cm Mini-Protean II BioRad electrophoresis system (BioRad, Hertfordshire, U.K.). Labelled ODNs were boiled for 5 minutes at 90°C in 10µl of loading buffer (90% formamide, 0.1% bromophenol blue and 0.1% xylene blue, all Sigma-Aldrich, Poole, U.K) and loaded separately onto

a 20cm denaturing 20% polyacrylamide gel (containing 7M urea). The quantity of labelled ODN loaded onto the gel for electrophoresis was determined by the amount of radioactivity (counts per second) registered on a hand held Gieger-counter (Morgan Mini-Instruments Ltd., Essex, U.K.). Equal numbers of counts for each ODN were loaded per well of the gel which was electrophoresed at 30mA for 2.5 hours in tris-borate-EDTA buffer (Sigma-Aldrich, Poole, U.K.).

Both bromophenol blue and xylene blue dyes allowed estimation of the migration rates of the ODNs down the gel (Sambrook *et al.* 1989). Autoradiography was used to visualise the separated ODN bands, gels being exposed to autoradiography film (Amersham, Buckinghamshire, U.K.) for 12 hour in the dark, at -70°C.

3.2.5 MANNOSYLATED AND NON-MANNOSYLATED BSA-FLUORESCEIN BINDING

Mannosylated-BSA-fluorescein and non-mannosylated BSA-fluorescein conjugates were a gift from Dr. A. C. Roche (Cellular and Molecular Glycoconjugate Laboratories, Orleans, France), provided as 1mg of solid (-20°C). Binding of these fluorescent species was assessed in both RAW264.7 and J774 macrophages, using the method described in section 2.7. Cells were incubated with 50μg of conjugate in 200μl serum free media for 1hour at 37°C and visualised under an inverted fluorescence microscope (Jenamed) using a green filter.

3.3 RESULTS

3.3.1 PHYSICAL ANALYSIS OF THE MONO-MANNOSYLATED OLIGONUCLEOTIDE CONJUGATE

3.3.1.1 Mass Spectrometric Analysis

As a means of verifying the conjugation of the mono-mannose phosphoramidite onto an ODN by the automated method described in section 2.2, a single nucleotide T-base was conjugated to the mannose derivative (section 3.2.2.2). The nucleotide in these columns is the first base of any ODN synthesised by the DNA synthesiser in 3'-end to 5'-end direction.

The T-mono-mannose-conjugate was prepared specifically for the purpose of analysing by electrospray ionisation mass spectrometry (section 3.2.3) due to its small size. The mass spectrometry trace produced is shown in figure 3.2.

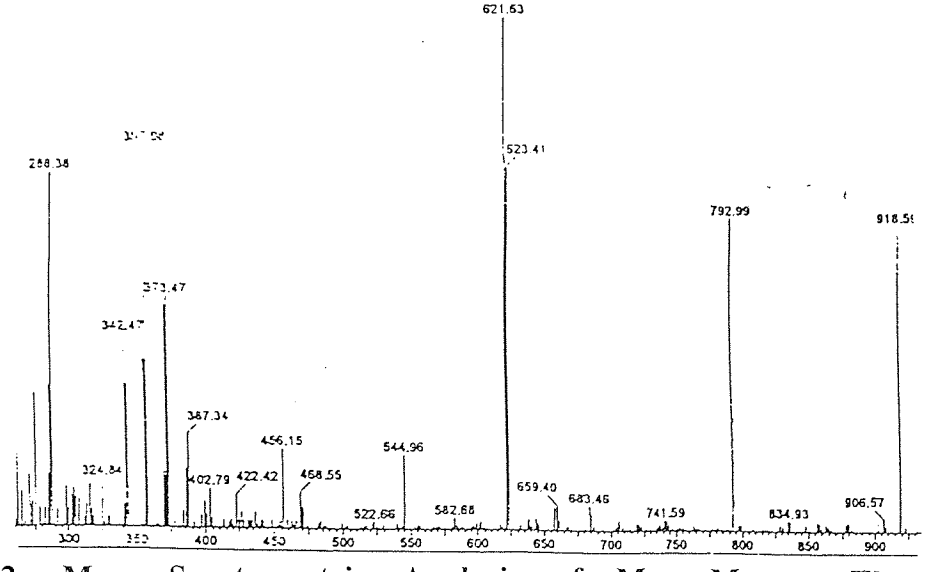


Figure 3.2: Mass Spectrometric Analysis of Mono-Mannose-Thymidine Conjugate.

The expected peak, indicating the molecular weight for the single nucleotide T-base conjugate can be seen along with impurities of varying size. Quantification of the conjugate was not possible from this method, therefore the efficiency of conjugation could not be determined. The mass spectrometric data shows that conjugation of a mannose residue to a nucleotide by the automated DNA synthesiser was successful. Therefore the mannose phosphoramidite derivative appears to maintain its integrity on the automated DNA synthesiser, enabling efficient conjugation. The presence of contaminants in the synthesis mixture is also shown by mass spectrometric analysis. Such contaminants do not pose a problem however when the mono-mannose conjugate is used in cellular association studies, the process of radiolabelling (section 3.2.4) and purification of labelled ODNs by PAGE is such that only a discrete band of a homogeneous ODN is purified.

3.3.1.2 Gel Shift Analysis

Conjugation of the mannose moiety onto a full length ODN (15D NOS) was verified by its electrophoretic mobility on PAGE. Two batches of the mono-mannose NOS ODN conjugate (referred to as mono-mannose conjugate) were synthesised on separate occasions, from the mono-mannose phosphoramidite (section 3.2.1), identical synthesis and purification methods were followed for each synthesis. These conjugates (designated batches I and II) were 3'-radiolabelled (section 3.2.4) along with unconjugated 15D NOS and 16D NOS. All ODNs were electrophoresed on a 20% denaturing polyacrylamide gel, this high percentage acrylamide gel having a high resolution for distinguishing single nucleotide differences between ODNs. The electrophoretic mobility of ODNs on denaturing gels is a direct function of size.

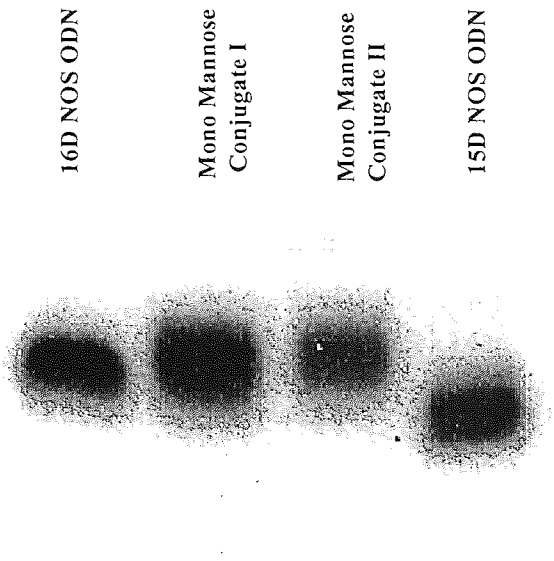


Figure 3.3: Characterisation of Mono-mannose Conjugated Oligonucleotide by Electrophoretic Gel Mobility Assay. Autoradiography of ^{32}P- labelled ODNs electrophoresed by 20% denaturing PAGE.

As shown by figure 3.3, it is clear that both batches of mono-mannose conjugate co-migrated with the 16D unconjugated ODN, with a slower mobility than the unconjugated 15D ODN. From this we can deduce that conjugation on the synthesiser was successful. This data combined with that produced by mass spectrometry of the T-mono-mannose conjugate (section 3.1.1.1) proves the conjugation of a mono-mannose group to the 15D ODN using our novel synthesis methodology. All subsequent experiments performed used these two batches of the mono-mannose conjugated ODN.

3.3.2 Binding of a Mannosylated-BSA-Fluorescein Conjugate to J774 and RAW264.7 Cells

The two murine macrophages cell lines, RAW264.7 and J774 were evaluated for their capacity to bind a mannosylated species. A neoglycoprotein (synthetic glycosylated protein), mannosylated-BSA-fluorescein and a control BSA-fluorescein species (non-mannosylated) were used as ligands (section 3.2.5). Uptake into the cell lines and where possible subcellular distribution of these species was studied (figures 3.4 and

3.5). The results were recorded photographically using an inverted fluorescence microscope, following the protocol described in section 2.7. At the time of these experiments accurate quantification of fluorescence on the cells, as a measurement of the amount of ODN bound, was not possible, as we did not have access to relevant equipment such as confocal microscopes. Therefore, the resultant fluorescent intensity in each cell line for each type of fluoresceinated-BSA species was visually assessed.

A series of preliminary studies were performed to optimise the incubation period, the concentrations of fluoresceinated-BSA species and the microscope settings (data not shown). A standard protocol was established as described in section 2.7.

The result of 4hr incubation at 37°C with each of these fluoresceinated species are shown in figures 3.4 (J774 cells), 3.5 (RAW264.7) and 3.6 (J774 and RAW264.7 cells under phase contrast). Determining sub-cellular distribution was not possible due to poor resolution, the photographs produced from 100x magnification not being very clear due to the particular type of microscope used.

J774 cells bound a noticeably higher degree of the mannosylated-BSA-fluorescein species (figure 3.4A) than the control BSA-fluorescein (figure 3.4B). There is clearly a higher degree of fluorescence on J774 cells incubated with the mannosylated-BSA-fluorescein species, which must be attributable to the mannose groups, as mannosylation is the only difference between the two species. Fluorescence is concentrated around the periphery of the cells, which indicates that binding was mainly to the surface of the cells. There appears to be a punctate distribution of fluorescence within the cells, which is indicative of fluoresceinated species being internalised into vesicles within the cell, hypothesised to be via endocytosis. However, a specialised microscope with a higher resolution would be needed to visualise this pattern more clearly.

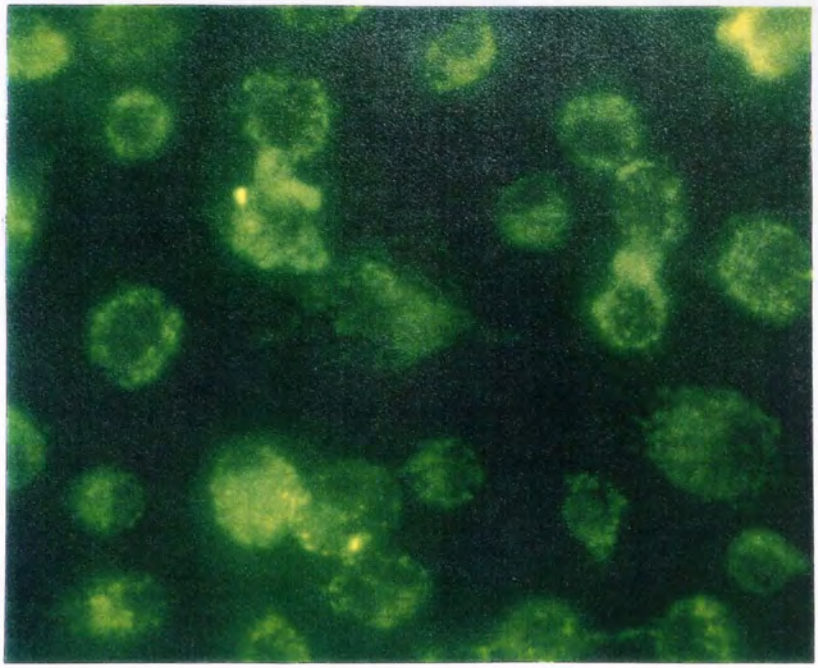
Micrographs of RAW264.7 cells incubated with the BSA-fluorescein species are shown in figures 3.5A, B, C and D. Figures 3.5A and B were taken at a lower magnification (25x) than 3.5C and D (100x), both highlighting the different degrees of

cellular association for the two species. As with the J774 cells, there was a significantly higher degree of fluorescence on RAW264.7 cells incubated with the mannosylated-BSA species compared to the control species. The higher magnification distinctively shows the differing quantities bound (figures 3.5C and D), however giving no more an indication of sub-cellular distribution. Fluorescence is again seen mainly on the periphery of the cells, in a punctate pattern, with a dark region in the centre, which is probably the nucleus, where little or no fluorescence is seen.

The cells for each experimental compound were at a similar level of confluence in each case. Figure 3.6A and 3.6B shows typical cell monolayers of RAW264.7 cells used in the experiments. Therefore the differing degrees of fluorescent intensity seen in each photograph can be attributed to binding alone, as cells were the same densities in all chambers. A trypan blue exclusion assay was used to determine toxicity of the BSA conjugates, no evidence of cytotoxicity was seen (data not shown).

Therefore we can conclude that the mannose moiety enhances binding of BSA to both macrophage cell lines, possibly by a mechanism which results in internalisation of BSA into endocytic vesicles. Information on subcellular distribution of the ligand or distribution of mannose receptors cannot be inferred from this data, but it is clear that J774 and RAW264.7 cells both have an affinity for binding mannose conjugated species. It has to be noted that these BSA species were conjugated to twenty mannose residues which will confer very high affinity binding as the mannose receptor binds species with multiple mannose residues more avidly than species with few residues (section 3.1.5). The ODN conjugate synthesised, possessing only one mannose residue, differs significantly from these BSA conjugates. The chemistry involved in mono-mannose conjugate synthesis was difficult (section 3.2.2.2), therefore preliminary studies necessitated single mannose group conjugation. This study highlights the ability of these macrophage cell lines to bind mannosylated species, proving to be a good model for binding mannosylated ODNs.

A



B

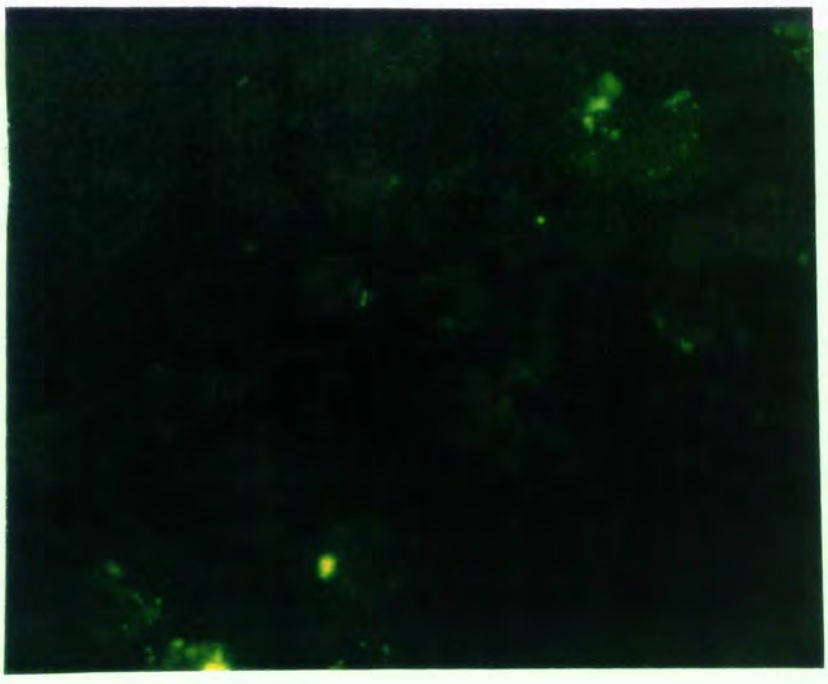


Figure 3.4 (A and B). Fluorescent Micrographs of J774 Cells 1.25 x 105/ml cells seeded per chamber 24 hrs prior to 4hrs incubation with (A) Mannosylated-BSA-fluorescein. (B) Control BSA-fluorescein, following which cells were washed and visualised by a Jenamed fluorescence microscope (50x).

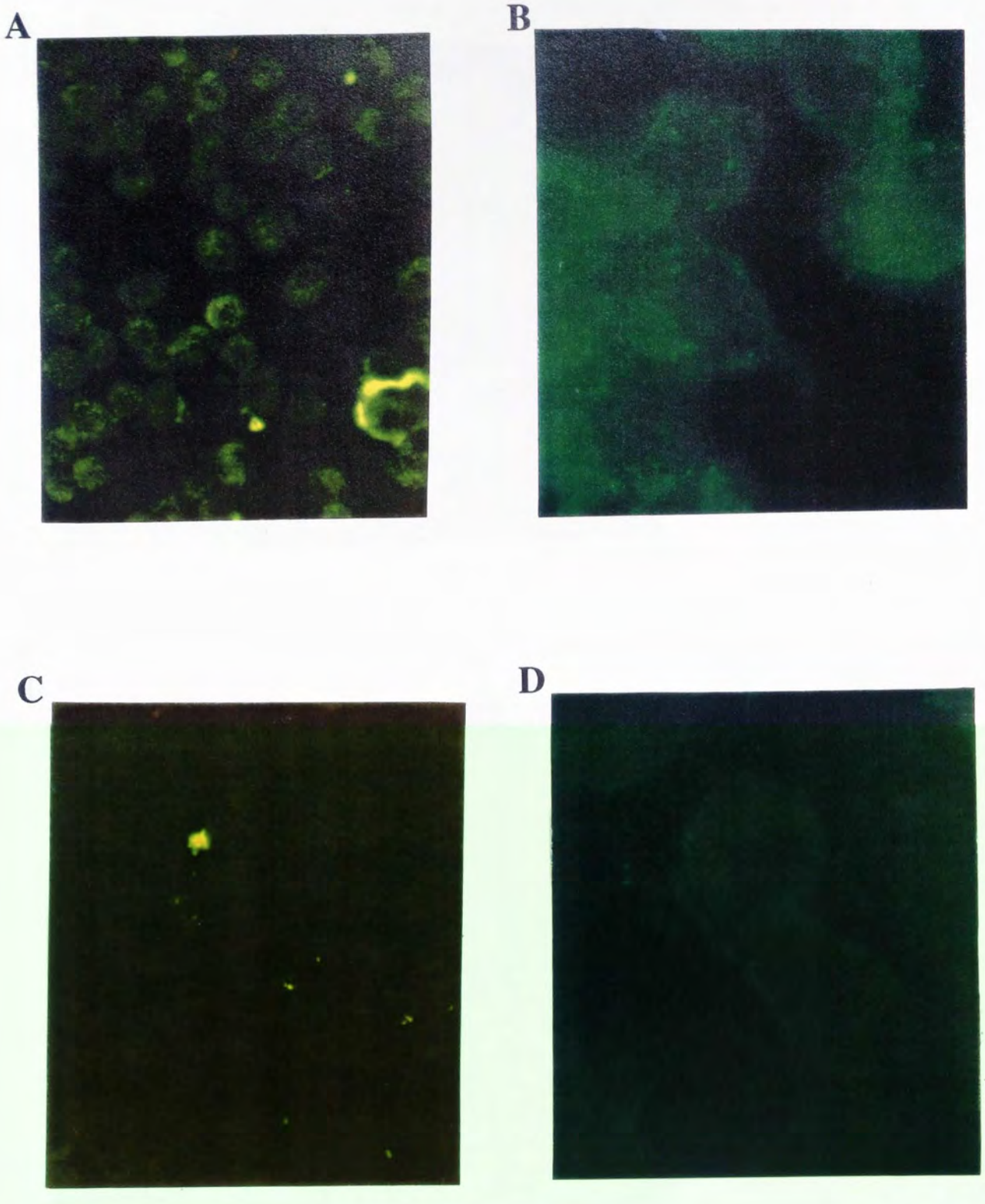
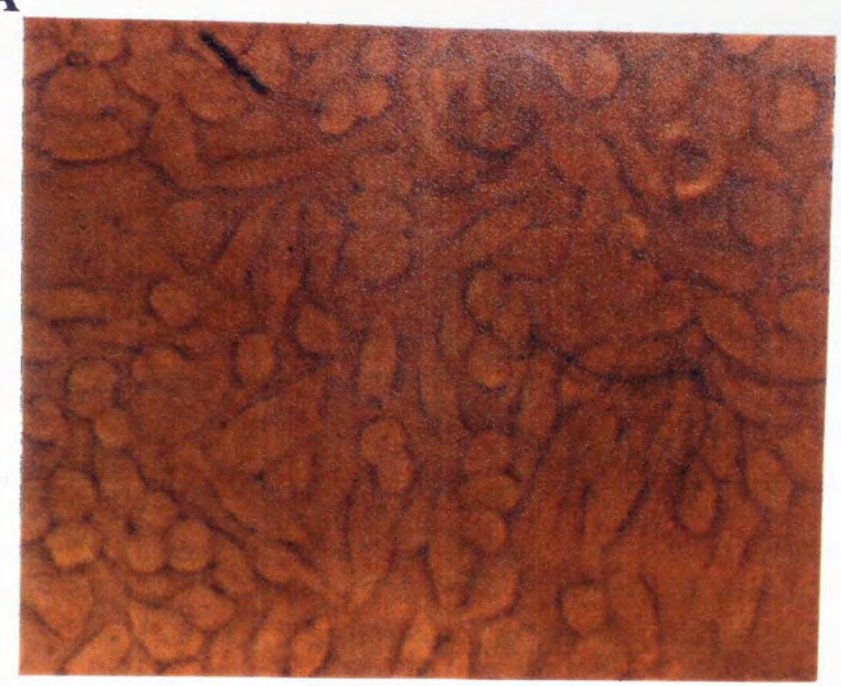


Figure 3.5 (A, B, C and D). Fluorescent Micrographs of RAW264.7 Cells 1.25 x 105/ml cells seeded per chamber 24 hrs prior to 4hrs incubation with (A and B) Mannosylated-BSA-fluorescein. (C and D) Control BSA-fluorescein, following which cells were washed and visualised by a Jenamed fluorescence microscope (A and C at 25x and B and D at 100x).

A



B

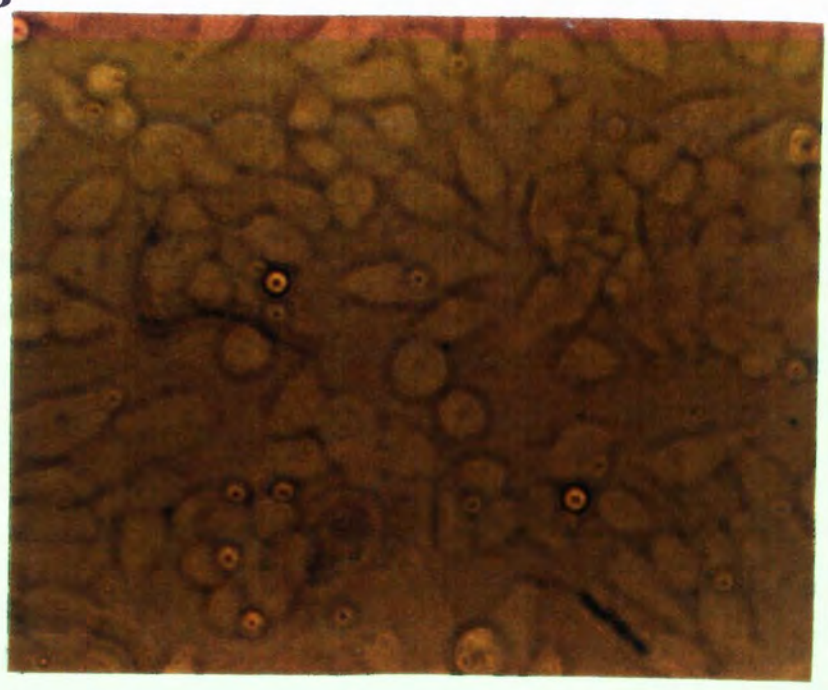


Figure 3.6 (A and B). Phase Contrast Micrographs of RAW264.7 Cells. 1.25 x 105/ml cells seeded per chamber 24 hrs prior to 4hrs incubation with (A) Mannosylated-BSA-fluorescein. (B) Control BSA-fluorescein, following which cells were washed and visualised by a Jenamed fluorescence microscope (25x).

3.3.3 CELLULAR ASSOCIATION OF THE MONO-MANNOSE CONJUGATE

As described earlier, the cellular association and uptake of ODNs is greatly influenced by a many factors, including the ODN size and chemistry, conjugation of side groups, exogenous conditions such as pH and temperature, and the target cell type.

The cellular association characteristics of the mono-mannose conjugate and unconjugated control ODN were investigated in RAW264.7 macrophages, in order to ascertain whether the mannose moiety enhanced cellular association. The influence of various exogenous agents and conditions such as temperature, metabolic inhibitors, presence of competitors and endocytosis disrupting agents were also evaluated. Comparisons of cellular association were also made between different cell lines, J774 and RAW264.7 macrophages and the human glioblastoma cell line U87-MG.

3.3.3.1 The Stability of Oligonucleotide Conjugates in Cell Culture

The existence of cellular nucleases, both exonucleases and endonucleases, lead to the degradation of ODNs into shorter fragments. These shorter fragments and ^{32}P radioisotope can become bound and internalised by cells, distorting cellular association results (section 2.4). The synthesis of ODNs with differing backbone types and conjugate moieties makes prediction of their response to cellular nucleases difficult. Unconjugated, unmodified phosphodiesters are particularly susceptible to such degradation.

Degraded ODN fragments, generated by nuclease action, can potentially possess the conjugated moiety, the label (radioisotope, fluorescent) or neither. Therefore, cellular association data may reflect the behaviour of a heterogeneous mixture of ODN species. To assess the degree of ODN degradation, in the serum free extracellular medium (D-MEM) of J774, RAW264.7 and U87-MG cells, the degradation profile at 37°C over time was evaluated. Serum free medium was used to minimise degradation, as this is the source of nucleases in culture medium, however, over time, the cells

themselves secrete nucleases. The label used in these studies, 3'-end ^{32}P radioisotope, enabled analysis of degradation products by gel electrophoretic separation from intact ODN. ODNs of different sizes migrate at different rates. Quantification of the individual degradation bands was performed by densitometry of autoradiographs (section 2.3.4).

Figures 3.7, 3.8 and 3.9 above show the stability profiles for the mono-mannose-conjugate and unconjugated ODNs in the cell lines J774, RAW264.7 and U87-MG respectively. Conjugates I and II (section 3.1.2) denote two separate syntheses of the mono-mannose-conjugate, the syntheses being performed on the same day using the same batch of the mannose phosphoramidite. Only the stability of conjugate I was assessed in U87-MG cells due to the limited quantities of the conjugate available.

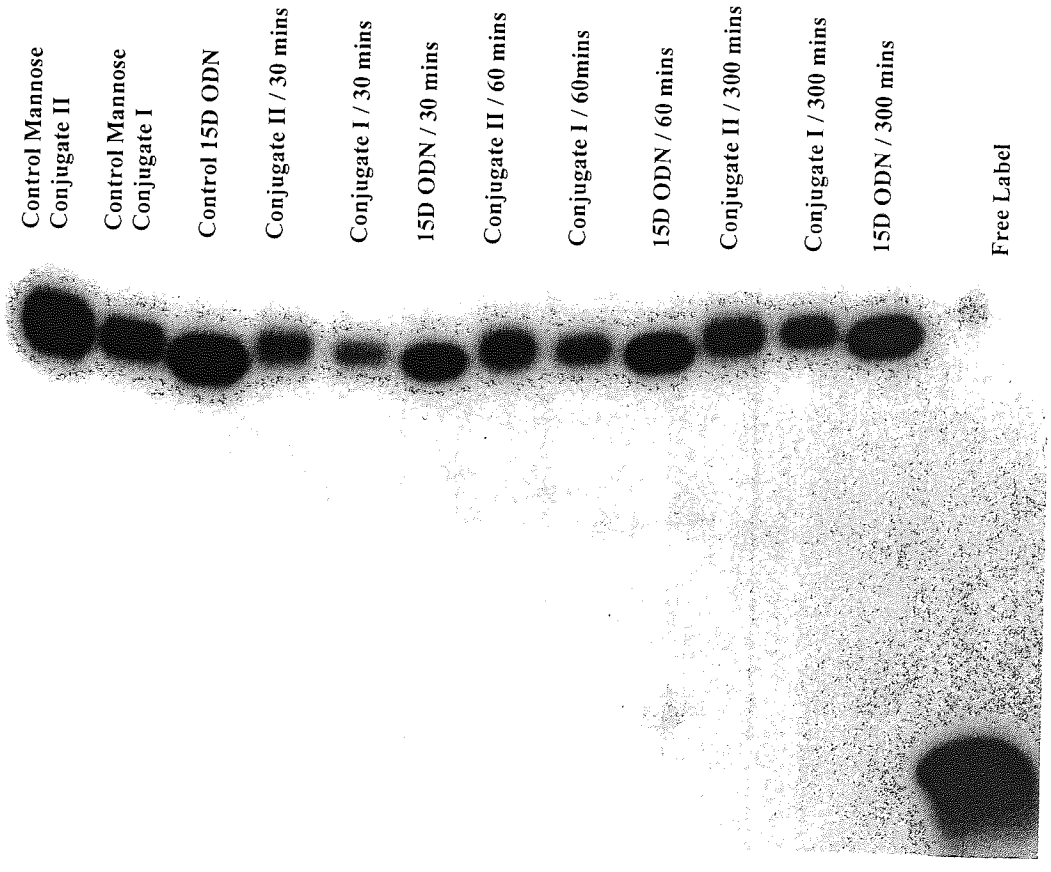


Figure 3.7. Stability of Mono-mannose conjugate and unconjugated 15D ODNs incubated with J774 cells, over time at 37°C. Autoradiography of 3'-end ^{32}P-labelled ODNs electrophoresed by 20% denaturing PAGE.

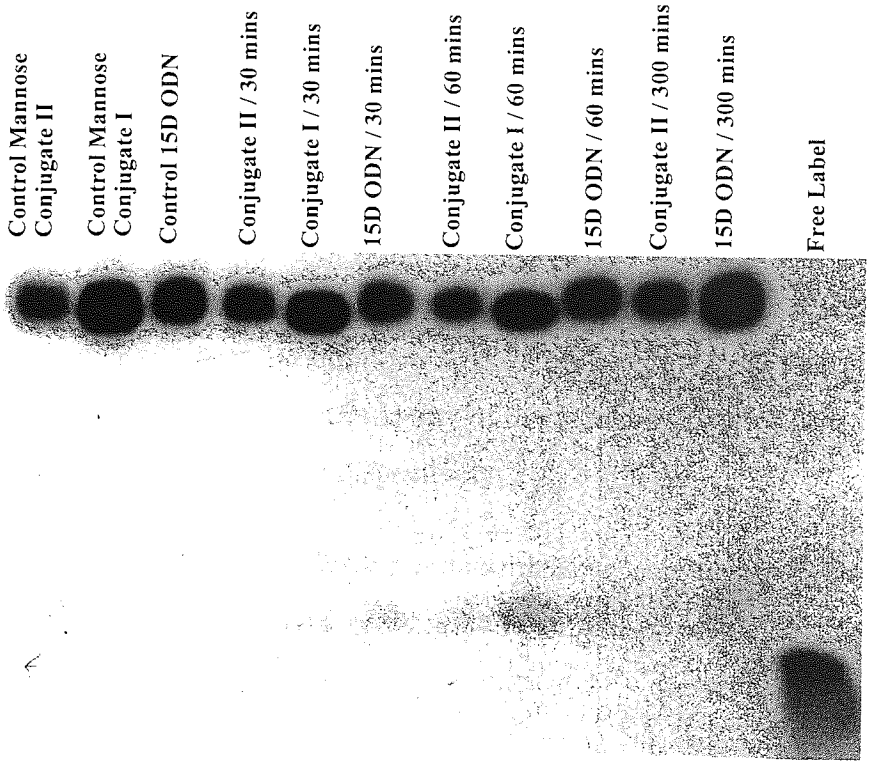


Figure 3.8. Stability of Mono-Mannose conjugate and Unconjugated 15D ODNs incubated with RAW264.7 cells, over time at 37°C. Autoradiography of 3'-end ^{32}P-labelled ODNs electrophoresed by 20% denaturing PAGE.

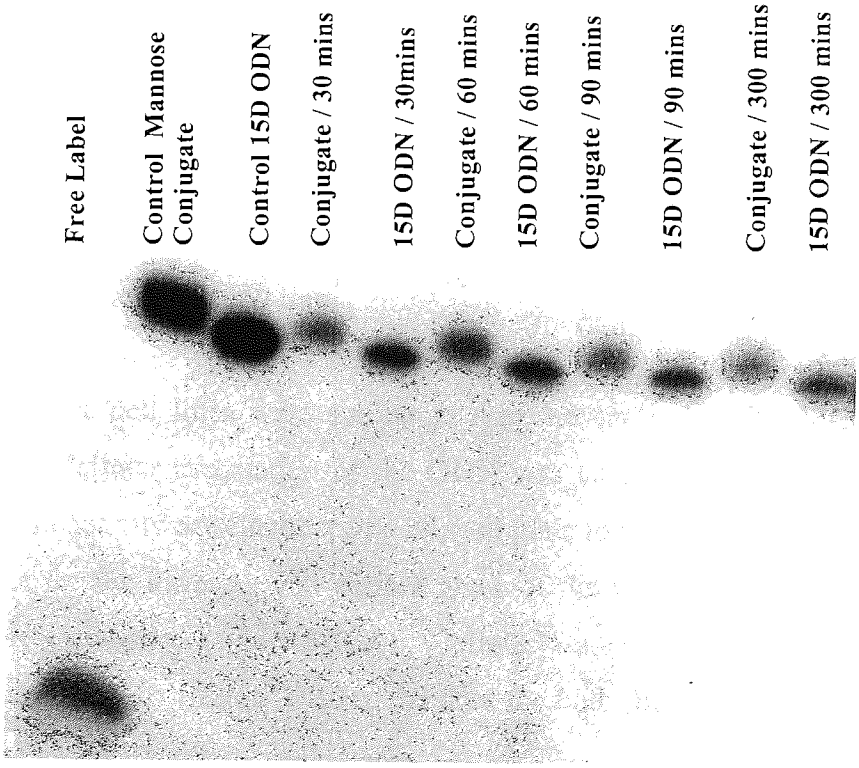


Figure 3.9. Stability of Mono-mannose conjugate and Unconjugated 15D ODNs incubated with U87-MG cells, over time at 37°C. Autoradiography of 3'-end ^{32}P-labelled ODNs electrophoresed by 20% denaturing PAGE.

For all three-cell lines (figures 3.7, 3.8, and 3.9) both the mono-mannose-conjugate and 15D ODNs are very stable in serum free medium when incubated at 37°C. Shorter ODN fragments or traces of free radiolabel would be detected by 20% PAGE, bands being seen adjacent to the free label (^{32}P-ATP) band. As shown by figure 3.8, RAW264.7 cells degraded the 15D ODN after 300 minutes and 60 minutes incubation, a faint band adjacent to the free label was seen (lanes B and E respectively). A small amount of free label was seen for both conjugates I and II at 300 minutes (figure 3.8). No degradation was seen for any ODN at any of the incubation periods for either J774 or U87-MG cells, figure 3.7 and 3.9 respectively.

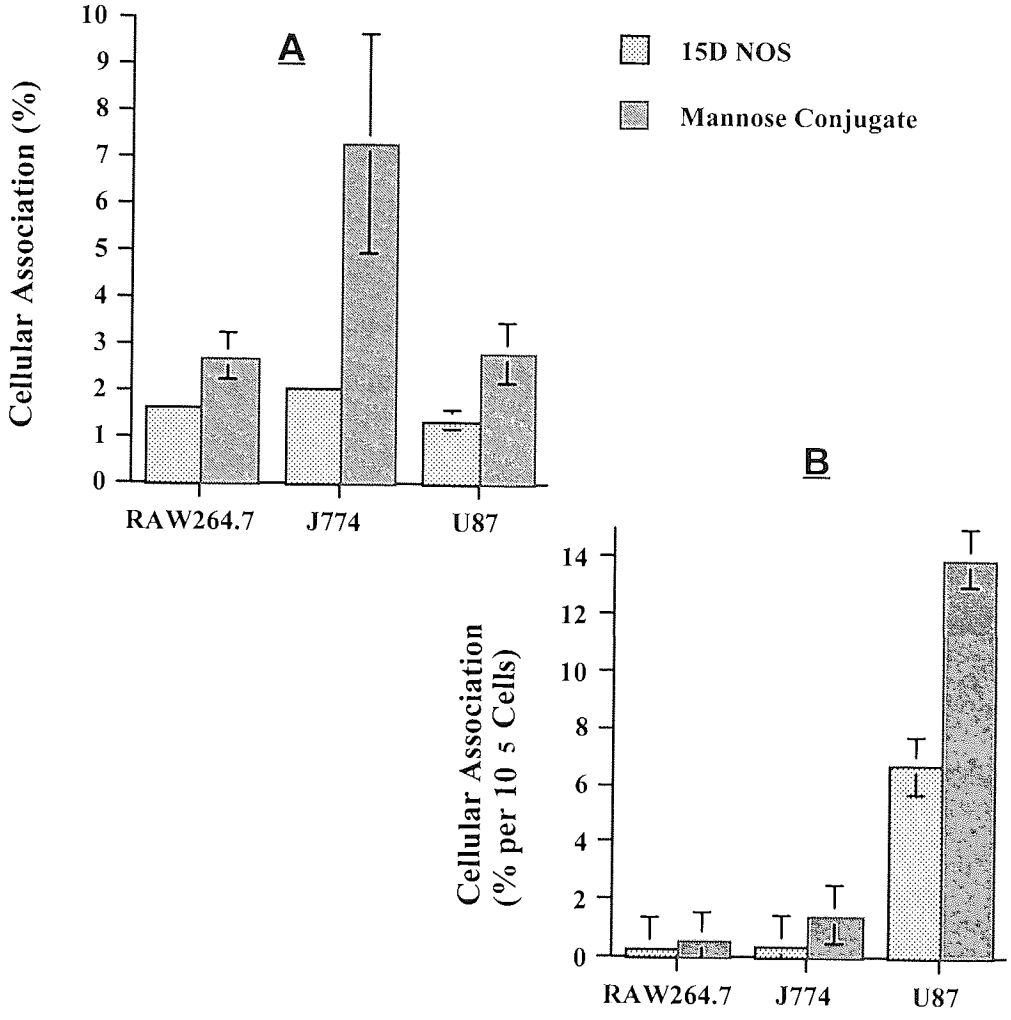
We can therefore assume that 15D and mono-mannose conjugate ODNs that were shown to be bound and internalised in both the macrophage cell lines, in subsequent studies, were intact full length for up to 300 minutes at 37°C in serum free medium.

3.3.3.2 Comparison of Cellular Association in Different Cell Lines.

Comparisons of cellular association for the mono-mannose conjugate and control unconjugated 15D ODN were made between three different cell lines, the murine macrophage cell lines RAW264.7 and J774 and the human Glioma cell line U87-MG. The experimental method described in section 2.5.2 was applied, using the cell densities specified for each cell line.

Both macrophage cell lines were similar in size, however the U87-MG cells were much larger. Cellular association of the ODN was calculated as the percentage of associated radioactivity per chamber of a 24 well plate to account for this difference in size and thus for the differing cell surface areas of the cells, which would influence surface ODN association (figure 3.10A). Cellular association of the ODN was also calculated per 10^5 cells (figure 3.10B), the data for both plots derived from different experiments.

Figure 3.10 (A and B): Cellular Association of Mannose Conjugate and Unconjugated 15D Oligonucleotide by Different Cell Lines.
Graph to show the cellular association (%) of mono-mannose conjugate and 15D NOS ODNs by different cell lines (RAW264.7, J774 macrophages and U87-MG glioma cells), expressed as % per well of cells (A) and per 10^5 cells (B), following 3hrs incubation at 37°C. n=3, bars=SD



Mannose receptors are not found on the plasma membrane of all cell types, hence it was expected that the two macrophage cell lines would bind the conjugate to a greater extent than the glioma cells. However this was not found to be the case (figure 3.10B) U87-MG (10^5) cells bound both the mono-mannose conjugate and the control 15D ODN to a greater extent than the same number of either of the macrophage cell lines. This could be attributed to a number of possibilities. The high passage number

(section 2.1.2) of the U87-MG cells, passage number 127, was considerably more than both the RAW264.7 and J774 cells (passage numbers 48 and 62 respectively). The characteristics of cells cultured *in vitro* alter over time, adopting unusual characteristics not found on the native cell line. The plasma membrane may loose its integrity becoming more porous or its constituents (proteins, glycoproteins or lipids) may be expressed to a different degree. There was a statistically significant difference in cellular association between of the 15D ODN and mono-mannose conjugate in all three cell lines after 3hr incubation at 37°C, figure 3.10A, ($p=0.018$, $p=0.017$ and $p=0.018$ for J774, RAW264.7 and U87-MG cells respectively). The relative increases in cellular association from unconjugated to mono-mannose conjugate, after 3hr incubation at 37°C, was greatest in J774 cells by approximately 3.5-fold compared to 0.6-fold for RAW264.7 cells and 2-fold for U87-MG cells.

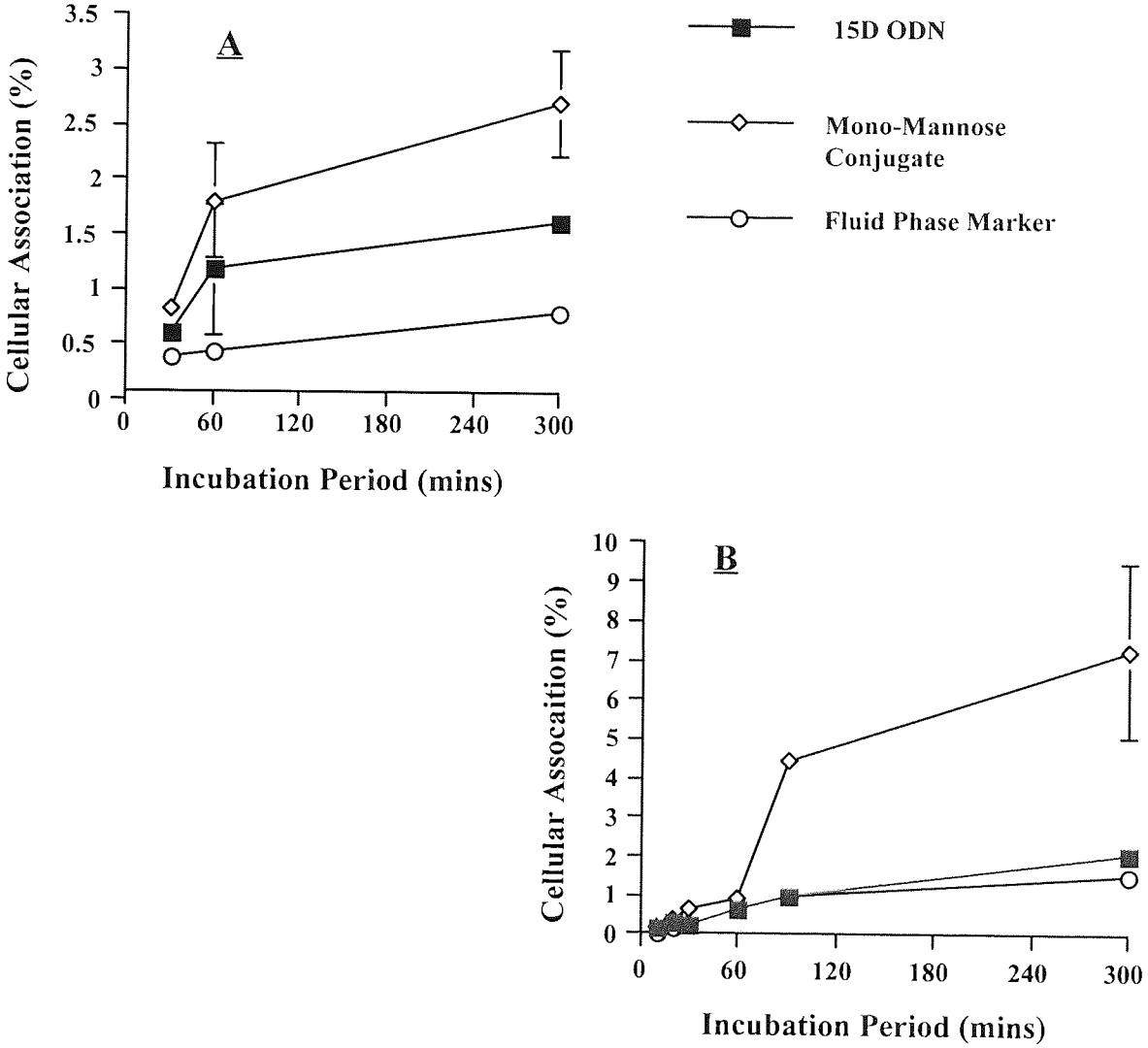
Also U87-MG cells are larger in size than either J774 or RAW264.7 cells. This is reflected in the fact that U87 cells are seeded at a density of 1×10^5 cells per chamber of a 24 well plate, whereas both RAW264.7 and J774 cells are seeded at 5×10^5 cells per chamber. Therefore the differences in binding of the mannose-conjugate and control 15D ODNs may not reflect the number of mannose receptors or the uptake of the conjugate but differing rates of fluid phase endocytosis on the surface of the cell (section 1.8.2). U87-MG cells have a higher surface area and so higher plasma membranes surface area per cell than macrophages. The data obtained for cellular association, when the cell number is normalised to 10^5, is therefore not truly comparative. A further experiment would be to express ODN uptake after normalising for protein content of the cell, protein content in part accounting for the differing surface area of cells and therefore differing levels of cell surface proteins (such as receptors). Cellular association of the mono-mannose conjugate was higher than that of the unconjugated control 15-ODN in all three cell lines following 3 hours incubation. Therefore it appears that the cellular association of the ODN is enhanced when conjugated to the mannose residue. This data may also implicate the presence of mannose receptors in U87-MG cells, although further studies are required.

3.3.3.3 Cellular Association Profiles Over Time in J774 and RAW264.7 Cells

The cellular association of the mono-mannose conjugate and control ODN was assessed at a number of time points over a period of five hours at 37°C in J774 and RAW264.7 cells, according to the general method described in section 2.5.2.2. The cellular association of these ODNs was compared to that of the fluid phase markers ^{3}H-labelled mannose and ^{3}H-labelled mannitol in J774 cells and RAW264.7 cells respectively. Such markers are molecules, which do not specifically bind to the plasma membrane in order to gain access into the cell, but are passively internalised (Besterman *et al.* 1981).

As figures 3.11A and B shows, in J774 and RAW264.7 cells respectively, there is a significant difference between the binding profile of the mono-mannose ODN compared to that of the fluid phase marker ($P<0.0001$ for both cell lines after 300 minutes incubation), thus indicating that different mechanisms are involved. The greatest difference in binding is evident between that of the mono-mannose-conjugate and fluid phase marker. The high percentage cellular association of the mono-mannose conjugated ODN implies the existence of a more efficient method of binding/uptake, not attributable to the passive internalisation of extracellular fluid by pinocytosis. This is consistent with the fact that ODNs are large anionic molecules whose entry into the cell by pinocytosis is not favourable (Yukubov *et al.* 1989; Wu Pong *et al* 1994b). The significantly higher cellular association of the mono-mannose-conjugate in both macrophage cell lines may be attributable to mannose receptor binding and internalisation, such specificity not achieved by the unconjugated ODN. Therefore mono-mannose conjugation appears to enhance cellular association.

Figure 3.11 (A and B): Cellular Association of Mannose Conjugate and Unconjugated 15D Oligonucleotides over Time. Graph to show the cellular association (%) of antisense NOS control 15D, mono-mannose conjugate and a fluid phase marker in RAW264.7 cells (A) and J774 cells (B) over time at 37C. n=3, bars=SD

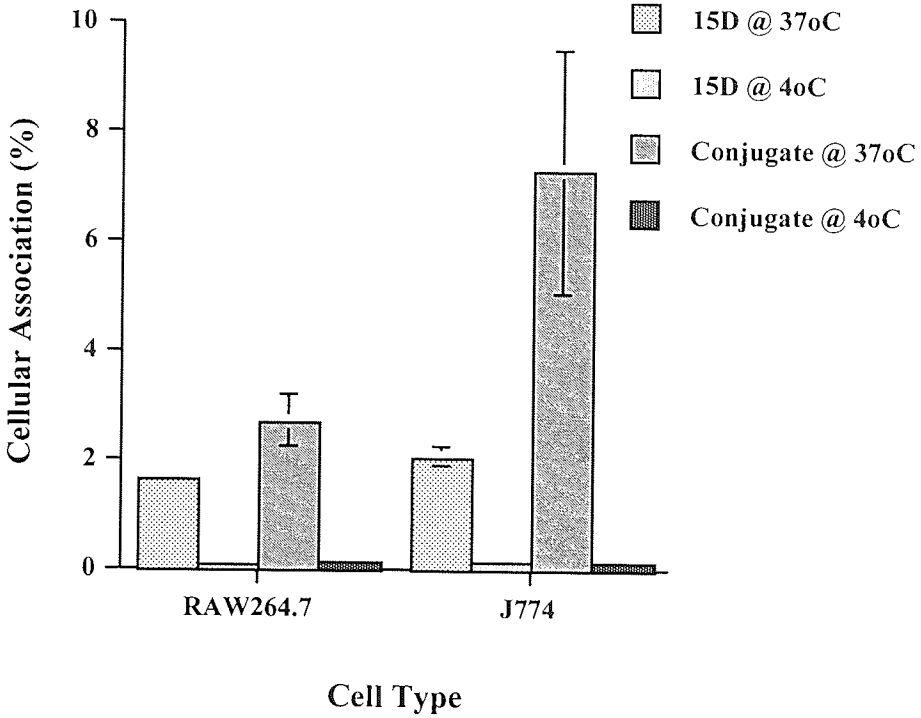


3.3.3.4 The Effect of Temperature on Cellular Association in J774 and RAW264.7 cells

To further evaluate the mechanism by which the control 15D and mono-mannose conjugated ODNs bind and enter macrophage cell lines the effect of temperature on this interaction was studied. As described in section 1.8.1 active processes such as RME, an essential component of mannose receptor binding, requires energy in order to bind and internalise large, charged molecules such as ODNs. The production of cellular energy, in the form of ATP, is dependent on the enzymatic action of ATP synthase (Lehninger, 1982). Enzymes are dependent on temperature, mammalian enzymes having optimal activity at 37°C and inactive at 4°C. A 10°C decrease in temperature can lead to the rate of a chemical reaction halving in some cases (Lehninger, 1982). In addition, lowering the temperature of cells could lead to a change in integrity or structure of the plasma membrane, therefore affecting the activity of membrane constituents (Wu Pong *et al.* 1992), some of which may be involved in exogenous ligand binding and internalisation. The cellular association of the ODNs was assessed at 37°C and 4°C in both macrophage cell lines following a 3 hour incubation period (figure 3.12).

The data demonstrates a very high degree of temperature dependency of both RAW264.7 and J774 cells for binding of both the 15D and mono-mannose conjugate ODNs (figure 3.12). There is a significant reduction in cellular association when the cells were incubated at 4 °C, for both the 15D control ODN and the mono-mannose-conjugate ($P<0.0001$ for both tpyes of ODN and in both cell lines). The amounts of ODN bound at 4°C are comparable to the values obtained for the fluid phase marker mannose and mannitol at 37°C (section 3.12 and figure 3.11), being the amount the cell associates with via a passive mechanism.

Figure 3.12: Effect of Temperature on Cellular Association of Mannose Conjugate and Unconjugated 15D Oligonucleotides.
Graph to show the effect of temperature on the cellular association (%) of 15D and mono-mannose-conjugate oligonucleotides in RAW264.7 and J774 cells following 3hr incubation (at 37oC and 4oC). n=4, bars=SD



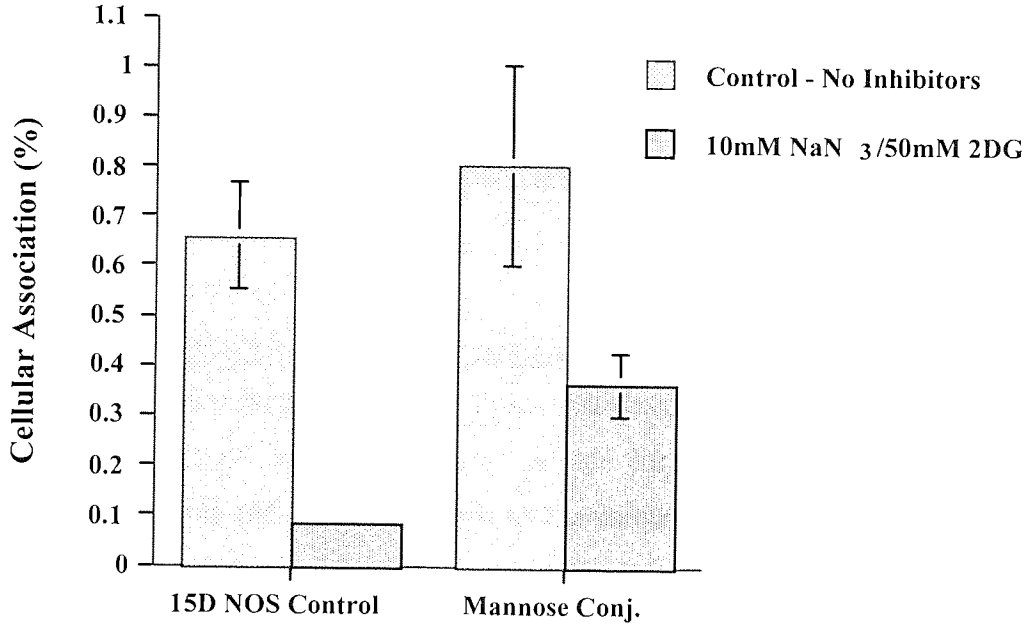
These findings are indicative of the involvement of active processes. Zamecnik et al. 1986 and Loke et al. 1989 report reductions in cellular association at 4 oC. Wu Pong *et al.* (1994b) observed cellular association of a 20mer phosphodiester ODN was reduced by 40% when incubated at 4 °C, compared to 37°C, the decrease attributed to lack of cellular energy. However, cellular association was not completely abolished by incubation at 4°C, this was attributed to cell surface binding of ODNs to membrane proteins by a passive mechanism or incomplete energy depletion.

3.3.3.5 The Effect of Metabolic Inhibitors on Cellular Association in RAW264.7 cells.

The dependence on temperature (as shown in section 3.2.5), implies an active process of ODN association with macrophages. To further investigate the cellular association

mechanism in RAW264.7 cells the effects of the metabolic inhibitor sodium azide (NaN_3) and 2-deoxyglucose (2-DG) (section 2.5.2.4) were studied. The method outlined in section 2.5.2.4 was followed, cells pre-incubated in the presence of inhibitors for 30 minutes before addition of the ODNs and further incubation at 37°C.

Figure 3.13: Effect of Metabolic Inhibitors on Cellular Association of Mannose Conjugate and Unconjugated 15D Oligoncleotides.
Graph to show the cellular association (%) of 15D NOS and mono-mannose conjugate oligonucleotides to RAW264.7 cells in the presence and absence of the metabolic inhibitors sodium azide (10mM) and 2-deoxyglucose (50mM) after 60 minutes incubation at 37°C. n=3, bars=SD



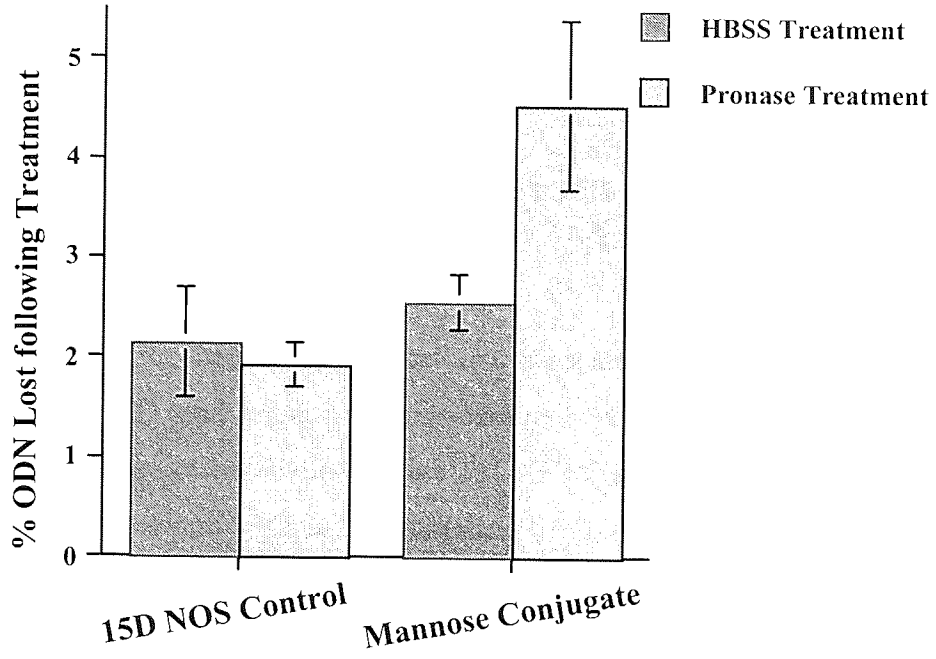
Significant reductions in cellular association in the presence of the inhibitors, 86.87% and 54.47% respectively, for the 15D and mono-mannose-conjugate ODNs (figure 3.13). Statistical analysis of the data was performed by a 'two-sample *t* -test' (section 2.6). Highly significant differences ($P<0.0001$ and $p=0.003$) between the presence and absence of inhibitors for the 15D and mono-mannose-conjugate ODNs respectively were observed. Again, as with the data obtained in section 3.3.3.4, there is a very high degree of energy dependency for the efficient association of ODNs.

Reduction in cellular association of the mono-mannose-conjugate not being as significant as for the 15D ODN in the presence of the inhibitors. It would be expected that if the mono-mannose conjugate were binding to a membrane receptor by an active process then it would be most affected by any energy depletion. A possible explanation could be that the mono-mannose conjugate may have a greater affinity for the cell membrane, and hence bind without the requirement of energy, compared to the 15D ODN. Although internalisation may be disrupted by the NaN_3 and 2-DG due to energy depletion, the cellular association on the outer surface may not be affected. As previously discussed energy depletion may not be total, a low level of energy production still occurring regardless of metabolic inhibitor treatment, therefore attributing to partial cellular association (Wu Pong *et al.* 1992). Again, the data points to an active, energy requiring process involved in oligo association with the cell, consistent with a receptor mediated mechanism.

3.3.3.6 The Effect of the Proteolytic Enzyme Pronase

The action of the proteolytic enzyme Pronase is described in section 2.5.2.6. To assess the degree of ODN bound to proteins on the surface of the plasma membrane, a Pronase wash step (at 4°C) was added to the standard protocol for assessing cellular association. The 15 minute incubation with the Pronase should remove any outer cell surface protein bound ODNs, the data therefore giving an indication of the amount of ODN binding outer surface proteins. The cellular association of the 15D and mono-mannose-conjugate ODNs were compared to a control in which the cells were incubated with HBSS. The data obtained is expressed as the amount of ODN lost in the wash step, figure 3.14.

Figure 3.14: Percentage Mannose Conjugate and Unconjugated 15D Oligonucleotides Lost following HBSS or Pronase Treatment. Graph to show the amount (%) of 15D oligonucleotide and mono-mannose conjugate stripped off RAW264.7 cells by a 15 min treatment with either HBSS or 0.25% Pronase/HBSS following an initial 1hr incubation at 37ºC. n=4, bars=SD



Approximately 63.9% of the mono-mannose-conjugate was removed by the Pronase compared to the control compared to 11.09% of 15D ODN under the same condition. Statistical evaluation revealed a significant difference in cellular association between a Pronase and a HBSS wash for both the 15D ODN (P=0.0088) and the mono-mannose conjugate (P=0.0158). The effects of Pronase, between the 15D and the mono-mannose-conjugate ODNs, was not as pronounced as would be expected if the mono-mannose conjugate were binding specifically, and with a high affinity, to the mannose receptor. Phosphodiesters do not bind proteins as avidly as phosphorothioates and therefore are expected not to be significantly affected by Pronase, which is the case for the 15D-control oligo.

Following Pronase treatment a greater percentage of the mono-mannose conjugate remained cellularly associated compared to the 15D ODN (figure 3.14), this portion possibly an internalised fraction. Therefore, significantly more of the mono-mannose-

conjugate is protein bound in comparison to the 15D control ODN. It can be assumed that the portion of ODN that remains bound to the cell after Pronase treatment is either very tightly bound to proteins, insensitive to the action of this proteolytic enzyme, bound to other non-protein plasma membrane moieties (lipids, glycolipids or glycoproteins) or internalised.

3.3.3.7 The Effect of Competition with Excess 15D Oligonucleotide and Mono-Mannose Conjugate

In order to determine specificity for cellular association to RAW264.7 cells, the binding of the 15D and mono-mannose-conjugate ODNs was compared in the presence and absence of unlabelled excess 15D ODN and mono-mannose-conjugate. A 250-fold excess of non-^{32}P-labelled (unlabelled) ODN was used (section 2.5.2.7). Cells were co-incubated with the labelled and unlabelled ODNs for 1 hour at 37°C.

The mono-mannose-conjugate associates with the RAW264.7 cells by an energy dependent mechanism involving cell surface proteins (sections 3.2.6 and 3.2.7). If binding of the conjugate to the cell is specific and a receptor is involved then this interaction should be such that it will not be affected by the presence of other extracellular moieties. Therefore, the cellular association of the mono-mannose-conjugate should not be affected by the presence of excess 15D ODN. Excess unlabelled mono-mannose-conjugate should however reduce the association of the labelled mono-mannose-conjugate due to competition for binding sites on the cell membrane, unless there is a large capacity for binding, and saturation is not reached. Likewise, labelled 15D ODN association to the cell should be reduced by excess unlabelled 15D ODN both labelled and unlabelled ODNs competing for the same sites. However, the binding of the labelled 15D may also be reduced by the presence of unlabelled excess mono-mannose-conjugate, the ODN portion of the conjugate could bind to the same site as the unconjugated 15D ODN, regardless of the mono-mannose moiety.

Figure 3.15: Effect of Competition on Cellular Association of Mannose Conjugate and Unconjugated 15D Oligonucleotides.
Graph to show the effects on cellular association (%) of 15D and mono-mannose conjugate ODNs on RAW264.7 cells of competition with 250-fold excess unlabeled 15D and mono-mannose conjugate ODNs after 1hr incubation at 37oC. n=4, bars=SD

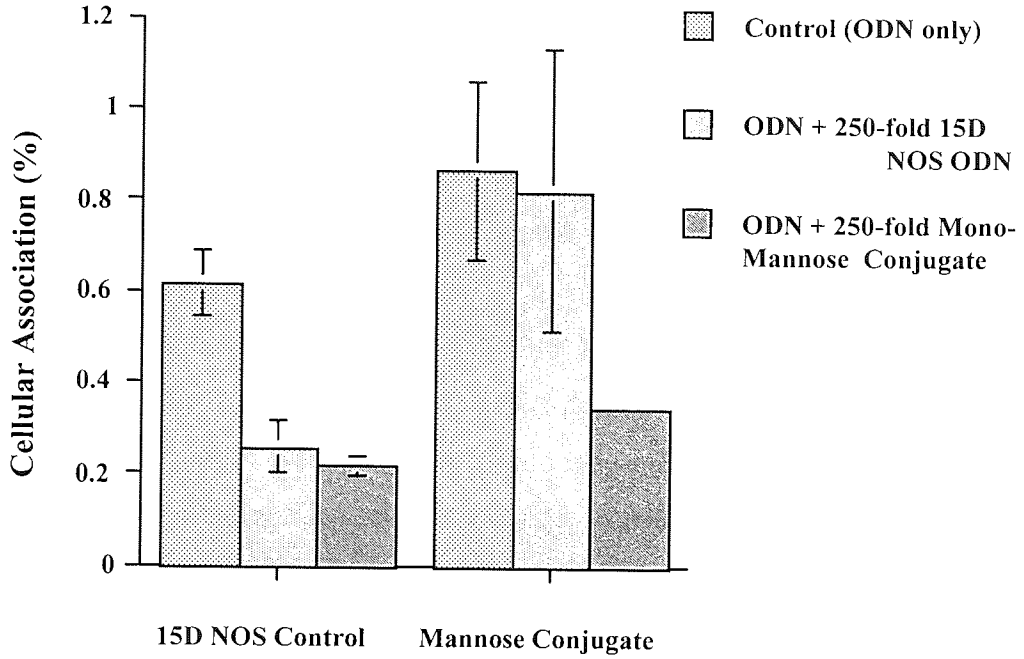


Figure 3.15 shows the effect of competition on the cellular association of each ODN. As expected, the presence of unlabelled excess 15D ODN does not significantly (P=0.4203). Affect binding of the mono-mannose-conjugate, causing only a 5.2% reduction in cellular association. Mono-mannose conjugate cellular association, in the presence of 250-fold excess unlabelled mono-mannose-conjugate, was however significantly reduced by 60.2% (P=0.0049). Therefore, in the case of the mono-mannose-conjugate, there is no cross competition with 15D ODN, but there is self-competition.

As expected both 15D and mono-mannose-conjugate compete for the 15D ODN. Cellular association of 15D was reduced by 58.5% and 64.6% in the presence of 250-fold excess of 15D and the mono-mannose-conjugate respectively, significant self and cross competition existing (P=0.0012 and P=0.0003 respectively).

The data indicates that the mono-mannose-conjugate binds specifically to the RAW264.7 cells, excess 15D ODN not competing for binding, however, there is a limited binding capacity on the cells, excess mono-mannose-conjugate competing with itself. Again, there is indirect evidence that the mono-mannose conjugate is binding to a specific entity on the RAW264.7 cell plasma membrane.

3.3.3.8 The Effect of Competition with Self, 15S and Free Mannose on Cellular Association of Mono-Mannose-Conjugate

Self-competition for the mono-mannose-conjugate has been established by the above experiment (section 3.2.8) using 250-fold excess of unlabelled ODN (25μM). Two lower concentrations of excess unlabelled mono-mannose-conjugate were assessed for competition for cellular association in order to determine the binding capacity of the RAW264.7 cells. If there was a plasma membrane binding protein or a specific mechanism involved in binding the mono-mannose-conjugate then the question of saturability needs to be addressed. Following the same protocol as above (section 2.5.2.7), self-competition was assessed using a 10-fold and 100-fold excess of unlabelled mono-mannose-conjugate.

Cross competition of the mono-mannose-conjugate with a 10-fold and 100-fold excess of phosphorothioate unconjugated NOS ODN, 15S (section 4.2.2) was also evaluated in RAW264.7 cells to determine binding specificity. Phosphorothioates bind to proteins non-specifically on the plasma membrane due to the nature of their structure, therefore they have the potential to bind to the same protein as the mono-mannose-conjugate. If the binding affinity of the protein for the mono-mannose-conjugate was not very strong then this binding could be reduced by non-specific binding to other moieties such as phosphorothioates. Finally, the effect of a 10-fold excess of unlabelled mannose sugar on the cellular association of the mono-mannose conjugate was also evaluated in RAW264.7 cells. These single monosaccharides should be able

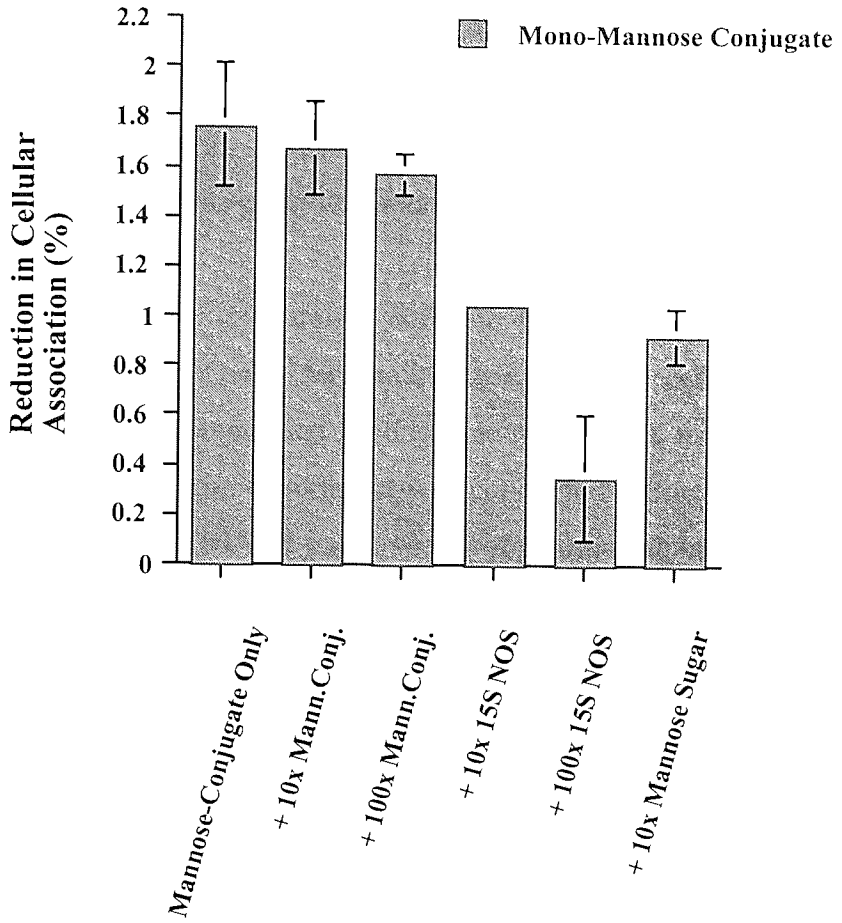
to bind to the same sites on the plasma membrane as the mono-mannose moiety of the conjugate, possibly inhibiting association.

As figure 3.16 shows, neither the 10-fold or 100-fold excess of unlabelled mono-mannose-conjugate had any significant effect on binding, 5.3% (P=0.3173) and 11.0% (P=0.1467) reductions in cell association respectively being seen, therefore no self competition at these concentrations. We can deduce from this data that, whatever site the conjugate is binding to or whatever mechanism employed, saturation has not been achieved over the concentration range used, the cell having the capacity to bind the 100-fold excess (10μM) of conjugate.

Significant competition is seen between the mono-mannose-conjugate and 15S ODN. The 15S ODN is either binding to the same sites on the plasma membrane as the mono-mannose-conjugate or binding to the conjugate itself preventing binding to the cell. At a 10-fold excess (1μM) of 15S NOS ODN a 40.7% (P=0.0042) reduction in cellular association is seen, an 80.1% reduction (P<0.001) is seen at a 100-fold excess. Phosphorothioates are known to bind non-specifically to proteins (Stein *et al.* 1992) therefore may bind the putative mannose receptor.

A 46.9% reduction in cellular association (P=0.0132) of the conjugate was seen in the presence of 10-fold excess of mannose monosaccharide, which is suprising. The free mannose may be able to act as a more efficient competitor of binding sites on the membrane due to its small size, not being sterically hindered. In the case of self-competition, conjugate molecules may bind to these membrane sites and become internalised, the receptor returning to the surface again for further binding (Tietze *et al.* 1982). Free mannose binding may not incite internalisation, the sites remaining blocked on the membrane surface, preventing further binding.

Figure 3.16: Effect of Cross Competition on Cellular Association of Mannose Conjugate and 15D Unconjugated Oligonucleotides.
Graph to show the cellular association (%) of the mono-mannose conjugate oligonucleotide in the presence of various agents in excess, after 1hr incubation at 37oC. n=3, bars=SD



Binding is significantly reduced by a phosphorothioate ODN, known to bind to proteins non-specifically. Therefore this indicates that there is a protein component involved in the mechanism, evidence for involvement of the mannose receptor. The binding affinity of the conjugate for this putative binding site or protein does not appear to be very strong, being competable with both a phosphorothioate ODN and free mannose monossacharide.

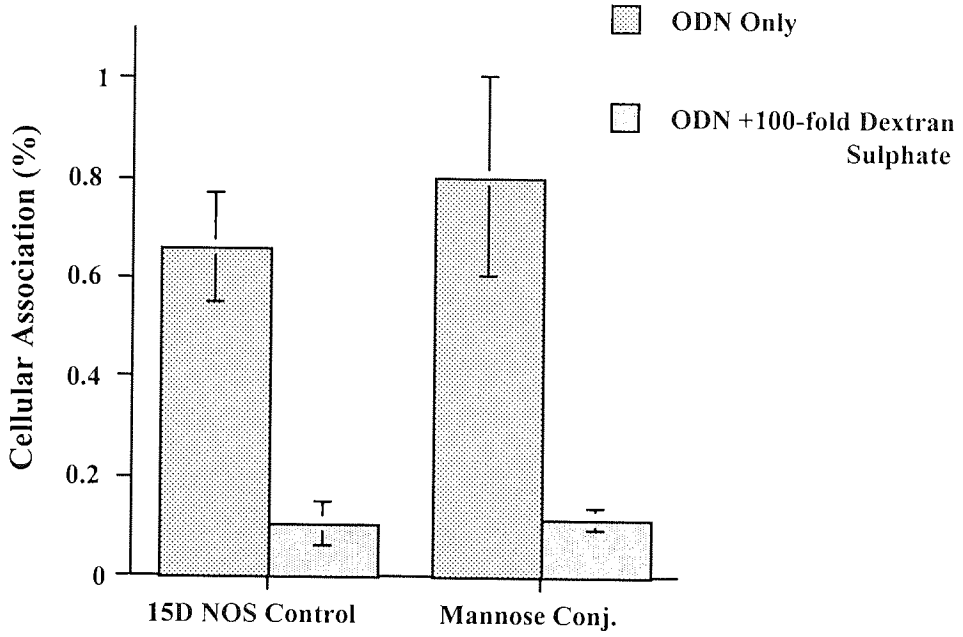
3.3.3.9 The Effect of Competition with Dextran Sulphate

In common with ODNs, a charged species of dextran sulphate was chosen as a polyanionic molecule, however differing in that it is a non-nucleic acid species. The negative charge of ODNs/polyanions and the positively charged surface proteins of the membrane have the potential to form an ionic interaction (an ionic bond). Due to its anionic nature dextran sulphate can potentially compete with ODNs for binding to the surface proteins of the plasma membrane.

If dextran sulphate is found to compete with both or either the 15D and mono-mannose-conjugate ODNs for binding to RAW264.7 cells we can conclude that the binding is due to competition for the same mechanism, namely ionic interactions with surface proteins. The experiment was conducted following the protocol outlined in section 2.5.2.8. A 100-fold molar excess of dextran sulphate was used, the RAW264.7 cells being pre-incubated with the polyanion for 15 minutes prior to addition of the ODN. Dextran sulphate is available in a number of different molecular weights. The one used in this study was selected to be of a similar M.W. to that of the ODNs, M.W of 5 000 because it was the nearest M.W. to the ODN, to eliminate any bias towards protein associating conveyed by a greater anionic charge.

The cellular association data obtained from competition between both the conjugated and control ODNs with dextran sulphate is shown in figure 3.17. The cellular associations of both the 15D and mono-mannose-conjugate ODNs was reduced by 86.7% ($P=3.88 \times 10^{-5}$) and 87.8% ($P=0.0002$) respectively, in the presence of 100-fold excess dextran sulphate. This polyanionic molecule appears to be interfering with the cellular association of both ODNs highly significantly. If the mono-mannose-conjugate were binding specifically and with a high affinity to a cell surface protein such as the mannose receptor, then we would expected its association to be unaffected by this polyanion, or a lesser extent as the 15D ODN.

Figure 3.17: Effect of Competition with Dextran Sulphate on Cellular Association of Mannose Conjugate and 15D Unconjugated Oligonucleotides. Graph to show the effects on cellular association (%) of 15D NOS and the mono-mannose conjugate ODNs by the poly-anion dextran sulphate (100-fold excess) in RAW264.7 cells 1hr incubation at 37C. n=4, bars=SD



A 48% reduction in cellular uptake of a 21-mer phosphodiester ODN is reported by Wu Pong *et al.* (1994b) in Rauscher erythro-leukaemic cells in the presence of 10μM of dextran sulphate. This data was interpreted as non-specific binding to the cells, as both DNA and ATP also competed with the 15D ODN, causing a reduction in uptake. A mechanism other than receptor mediated endocytosis is indicated for the phosphodiester, which may also be the case for the 15D ODN used in this study. Stein *et al.* (1993) report similar findings when the binding of phosphodiester ODNs were investigated in HL60 cells, leading to the conclusion that a pinocytic mechanism was operating. Their data showed that the phosphodiester ODN inhibited the internalisation of albumin, a fluid phase marker, providing evidence for passive internalisation of this PO ODN.

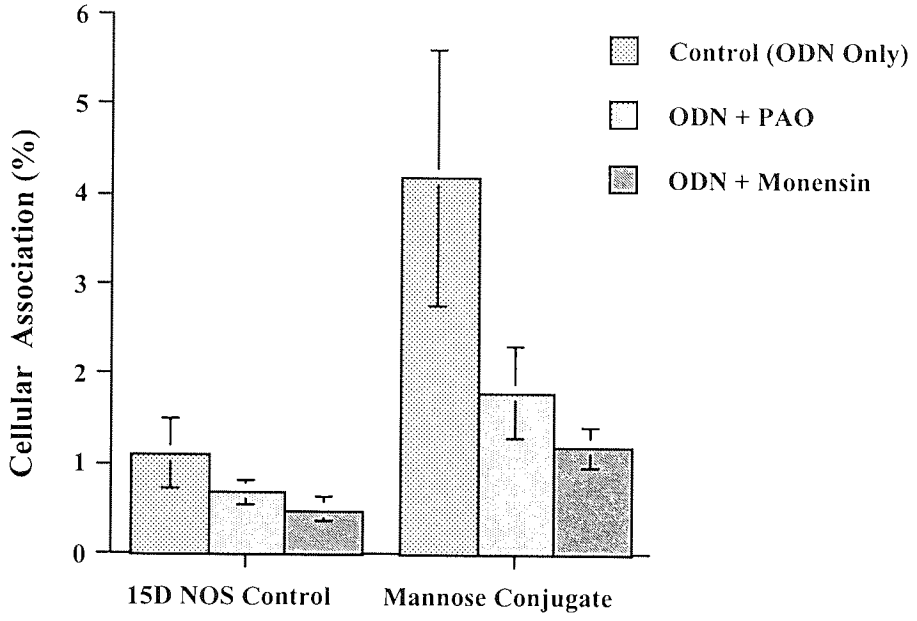
3.3.3.10 The Effect of Disrupting Endocytosis

As discussed in section 3.1.6 the mannose receptor binds its ligand, becomes internalised by a RME process and is recycled back to the surface after disassociation from its ligand. If the mono-mannose-conjugate is binding to the cell via the mannose receptor then disruption of this recycling process should lead to a decrease in ligand association. Phenyl Arsine Oxide (PAO) has been reported to be an inhibitor of RME for EGF and other ligands (Knutson *et al.* 1983; Hertel *et al.* 1985; Wu Pong *et el.* 1994b), described in section 2.5.2.10. Monensin is another such compound, used to disrupt the endocytosis cycle (Basu *et al.* 1981; Stein *et al.* 1984; Wileman *et al.* 1984; Wu Pong *et el.* 1992). Monensin works by preventing the dissociation of the ligand from the receptor (section 2.5.2.9), having other affects on the cell (Mollenhauer *et al.* 1990).

Cellular association of both the 15D and mono-mannose-conjugate ODNs was assessed in the presence and absence of PAO (100μM) and monensin (10μM) in RAW264.7 cells (sections 2.5.2.10 and 2.5.2.9 respectively). Cells were pre-incubated with PAO for 5 minutes and with monensin for 30 minutes prior to addition of the ODN followed by further incubation for 4 hours at 37°C. Figure 3.18 represents the effects of these agents on cellular association.

PAO reduced cellular association of 15D ODN by 39.0% (P=0.0019) and 56.9% (P=0.0123) for the mono-mannose-conjugate (figure 3.18). Monensin induced a greater reduction in binding, 15D ODN association being reduced by 56.4% (P=0.0105), and by 71.6% (P=0.0115) for the mono-mannose-conjugate (figure 3.). The data shows that the mono-mannose-conjugate was most affected by these endocytosis disrupters compared to the 15D ODN.

Figure 3.18: Effect of Endocytosis Inhibtors on Cellular Association of Mannose Conjugate and 15D Oligonucleotides. Graph to show the cellular association (%) of 15D and mono-mannose conjugate ODNs in RAW264.7 cells, in the presence and absence of the endocytosis disrupting agents Phenyl Arsine Oxide (PAO, 100uM)and Monensin (10uM). n=4, bars=SD



It is proposed that the mono-mannose-conjugate binds the mannose receptor on these macrophages, the resulting conjugate-receptor complex is internalised and the receptor recycled back to the surface for further ligand binding. If the recycling process is disrupted, and the number of receptors recycled back to the surface is decreased, then a corresponding reduction in further ligand binding would result.

The 15D ODN, theoretically not binding the mannose receptor, probably associates with DNA binding proteins which exist on the macrophage surface (section 1.8.3) (Bennett *et al.*. 1985; Loke *et al.* 1989). If this were the case and a similar RME pathway is involved in phosphodiester ODN uptake, then this binding protein would also be affected by these endocytosis disrupting agents in the same way, leading to a reduction in cellular association.

The precise mechanisms by which these agents work are unknown. Trypan blue cell viability studies (section 2.1.4) on the RAW264.7 cells in the presence and absence of PAO and monensin revealed no difference in viable cell number. Therefore any decreases in cell association of the ODNs cannot be attributed to a reduction in cell viability.

3.4 DISCUSSION AND CONCLUSIONS

We have recently described the synthesis of a novel mono-mannose ODN conjugate (Akhtar *et al.* 1996). Some stability and cellular association characterisitics of this novel compound *in vitro* was evaluated. To date no similar species have been described, whereby a mannose moiety has been directly conjugated onto an ODN, linked only by a six-carbon spacer arm. Previous studies have involved a protein component in the mannose ODN conjugate complex. We eliminated this component and hence reduced any adverse interactions and effects that proteins extracellularly induce, such as non-specific cellular association, steric hindrance of mannose binding to the receptor, induction of an immunogenic response or hindrance of internalisation of the complex due to its large size.

In general we expect the conjugate to behave like a conventional phosphodiester ODN. The greatest portion of the conjugate is the 15mer ODN moiety, comprising approximately 96% of the structure; the single mannose group therefore only represents a small component. These studies have partly characterised a mono-mannose conjugate and demonstrated a potential for delivery to macrophages. We ultimately aim to synthesise ODNs conjugated to multiple mannose residues to enhance this delivery.

Mass spec. analysis of a 1mer mono-mannose-T conjugate (section 3.1.1, figure 3.2) revealed that conjugation, performed directly on the DNA synthesiser, was successful. Demonstrating that this simple synthesis method, whereby a novel mannose phosphoramidite species was prepared and used as a conventional phosphoramidite, was successful. Electrophoretic mobility studies of the 15mer mono-mannose conjugate, compared to unconjugated 15D and 16D ODNs (figure 3.3), showed the conjugate to migrate in a similar manner to the unconjugated 16D ODN. Combining

the fact that the mass spec. data showed that mono-mannose conjugation to a T-base on the DNA synthesiser was successful, and the electrophorectic mobility studies showed that the 15D ODN migrated as a larger speices than the unconjugated 15D ODN, then it appears likely that mannose conjugation to an ODN is possible. Mass spec. analysis of the 15mer mono-mannose conjugate is required to verify conjugation. Both macrophage cell lines appear to preferentially bind mannosylated species, as shown by the micrographs produced when the cells were incubated with mannosylated-BSA-fluorescein or the control species BSA-fluorescein (figures 3.4 and 3.5, J774 and RAW264.7 cells respectively). J774 and RAW264.7 cells display a punctate distribution of the mannosylated-BSA-fluorescein species, implicating an endocytic mechanism of internalisation and an intra-vesicular distribution, consistent with the hypothesis that mannose receptor binding and internalisation is involved.

Both the conjugate and unconjugated control ODNs were found to be stable in the serum free medium of RAW264.7 and J774 macrophages and U87-MG cells, no degradation fragments were detected by analysis by PAGE (figures 3.7, 3.8 and 3.9). Therefore, these ODN species in all subsequent cellular association studies could be assumed as fully intact and undegraded.

From the uptake studies it is evident that there is a higher degree of cellular association of the mono-mannose-conjugate compared to the 15D ODN, especially at the longer incubation periods (figures 3.11), in both RAW264.7 and J774 cell lines. J774 cells were found to have a higher binding capacity for the mono-mannose-conjugate, 0.62%, 0.911% and 2.7% bound at 1, 3 and 5hr respectively, RAW264.7 cells binding 0.833%, 1.798% and 7.268% at the same time points. In both cell lines the binding profiles of the mono-mannose-conjugate and 15D ODNs are significantly higher than that of the fluid phase markers. Cellular association can therefore be attributed to a mechanism other than fluid phase endocytosis.

Cellular association for both ODNs was found to be highly temperature dependant, being significantly reduced at 4°C compared to that at 37°C (figure 3.12). Metabolic

inhibitors also significantly reduced cellular association of the mono-mannose-conjugate and 15D ODNs (section 3.2.6, figure 3.13) in both cell lines, compared to the cellular association values in the absense of the inhibitors. Combining the data from these two sets of experiments we can conclude that an energy dependant mechanism is involved for cellular association of both ODNs.

The proteolytic enzyme, Pronase, removed 63.9% of the mono-mannose conjugate from RAW264.7 cells compared to 11.09% of the 15D ODN, therefore indicating that a greater portion of the conjugate was cell surface protein bound compared to the 15D ODN (figure 3.14). This data is consistent with the theory that the mono-mannose conjugate binds the mannose receptor. Other researchers have performed similar studies when investigating the fraction of surface membrane protein bound ODNs. Wu Pong *et al.* (1992) used the proteolytic enzyme trypsin to strip off surface protein bound ODNs, sharing similar findings to ours in that all the ODN was not removed in the trypsin wash, 60% of the ODN remaining cell associated. Wu Pong *et al.* (1992) concluded that this fraction, unaffected by trypsin, was internalised by the cells. Beck *et al.* (1996) used Pronase to strip loosely bound ODNs from cultured human intestinal cells (Caco-2), and concluded that the fraction of ODN remaining bound to the cells was either internalised or tightly bound.

Competition studies revealed the ability of the conjugate to inhibit 15D ODN cellular association. Conjugate cellular association was not affected by excess 15D ODN (figure 3.15), therefore highlighting the specificity of the conjugate for binding to the RAW264.7 cells (section 3.2.8). The mono-mannose conjugate was also able to self-compete for cellular association, indicating a limited binding capacity, which is consistent with a receptor binding mechanism. 15S ODNs (figure 3.16) significantly reduced conjugate cellular association. A number of researchers have reported phosphorothioates binding non-specifically to cell surface proteins (Coulson *et al.* 1996), which may have been the cause of the reduction in cellular association seen, the 15S ODN possibly binding non-specifically to the mannose receptor. Mannose

monosaccharide (figure 3.16) was also seen to reduce conjugate binding, which may be a result of the mannose groups blocking the receptor.

The data obtained for competition of conjugate and 15D ODNs with the polyanionic molecule dextran sulphate showed similar reductions in cellular association (figure 3.17), approximately an 87% reduction for both ODNs. If mono-mannose conjugate were binding to a specific receptor on the surface of the cell then we would expect a lower degree of competition in the presence of dextran sulphate compared to the 15D ODN. Combined with the cross competition data with phosphorothioates and free mannose (figure 2.16), then it appears that conjugate specificity and affinity for the mannose receptor in RAW264.7 cells is not very strong, which can probably be attributed to the fact that there is only one mannose group conjugated to the ODN. As described in section 3.1.5, the mannose receptor optimally recognises molecules bearing multiple mannose residues, having the highest affinity and specificity for such species.

Disruption of the endocytic cycle of RAW264.7 cells with PAO and monensin resulted in the cellular association of the conjugate being more significantly reduced than the 15D ODN (figure 3.18). This data again implies that the mono-mannose conjugate binds by a mechanism similar to RME, possibly the mannose receptor. Wileman *et al.* (1984) reports the effects of monensin on alveolar macrophages, finding the RME of mannose terminated ligands was blocked by monensin due to entrapment of the ligand/receptor complex within lysosomal compartments of the cell. Monensin was stated to increase lysosomal pH, inhibiting mannose receptor recycling. Stein *et al.* (1984) reports a glycoprotein, which binds and internalises transferrin, the transferrin receptor. K562 cells, a human erythro-leukaemia line, which express this receptor, was investigated for transferrin binding in the presence and absence of monensin. The mechanism utilised by this receptor has been reported to involve internalisation of the ligand-receptor complex into an acidic compartment where dissociation of this complex occurs. The free transferrin receptor is then recycled back to the membrane surface for further ligand binding. In the presence of monensin

receptor recycling was inhibited in comparison to the recycling data obtained in the absense of monensin. A 50% reduction in number of transferrin receptors on the surface observed in the presence of monensin.

Teitelbaum *et al.* (1986) used PAO to investigate parathyroid hormone binding to its receptor in cultured bone cells, a reduction in association occurring on treatment, which was attributed to inhibition of RME. Wu Pong *et al* in 1992, 1994a and 1994b used PAO for the same function, for assessing the nature of ligand binding to cells in culture, phosphodiester ODN binding to erythro-leukaemia cells. PAO is classed amongst a group of molecules called trivalent arsenicals (section 2.5.2.10), reported to interact with an array of enzymes, including those involved in ATP synthesis causing an inhibition in ATP synthesis (Hertel *et al.* 1985). Therefore a depletion of cellular energy may be the cause of inhibition of cellular association as opposed to direct inhibition of endocytosis, although Hertel *et. al.* (1985) show ATP levels are unaltered in parallel to inhibition of the RME of epidermal growth factor and β-adrenergic receptors into human astrocytoma cells. The precise mechanisms by which these agents work are unknown. Trypan blue cell viability studies (section 2.1.4) on the RAW264.7 cells in the presence and absence of PAO and monensin revealed no difference in viable cell number. Therefore any decreases in cell association of the ODNs cannot be attributed to a reduction in cell viability.

Combining the data from all the various cellular association studies there is a strong indication that the mono-mannose conjugate binds to a greater degree and affinity to RAW264.7 macrophages compared to the 15D ODN. A receptor mediated mechanism may possibly be involved in binding although this initial data indicates that affinity may be poor for binding the mono-mannose conjugate. Multiple mannose residues on a ligand have been deemed as necessary for high affinity binding to the mannose receptor. The preliminary chemistry for mono-mannose conjugation to an ODN has now been developed (Akhtar *et al.* 1996) and will open up the possibility for conjugating mulitple mannose residues to an ODN. There is also the potential for synthesising a phosphorothioate mono-mannose conjugated ODN which would have a

longer $t^1/_2$ than the phosphodiester species in biological medium. Other sugar membrane lectins may also be conjugated to ODNs by synthesis of similar sugar phosphoramidites now that the preliminary chemistry has been established.

CHAPTER 4: LIPOPHILIC ODN CONJUGATES

4.1 INTRODUCTION

4.1.1 HIV INFECTION

The treatment of disease states caused by aberrant gene expression has been a major field of study with respect to antisense ODN therapy, being used for cardiovascular, auto-immune and nervous disorders. Viral genes are also a major target for antisense ODNs (Lemaitre *et al.* 1987; Hoke *et al.* 1991). Zamecnik *et al.* (1978) were the first to direct antisense ODNs to a viral target, Rous Sarcoma virus replication was inhibited by an antisense 13-mer phosphodiester ODN targeted to the 35S RNA of the virus in infected fibroblasts. Since this study, many other viral infections have been targeted for ODN therapy (Zamecnik *et al.* 1986; Lemaitre *et al.* 1987; Matsukura *et al.* 1989).

4.1.1.1 HIV Infection and AIDS

The first reported case of AIDS was in 1981. The most common form of AIDS in the industrial world is caused by a particular strain of virus called HIV-1 which was isolated and identified by Gallo *et al.* (1983) in the United States of America. A proliferation of studies have been directed towards investigating AIDS (Acquired Immune Deficiency Syndrome), which is caused by the Human Immunodeficiency Virus (HIV) in its final stages. HIV is a retrovirus, an RNA containing virus that transfers its genetic material into the DNA of its host, differing from other retroviruses because of the complexity of its genomic organisation (Demirhan *et al.* 1994),

containing five or six genes additional to *gag* (capsid proteins), *pol* (reverse transcriptase and integrase proteins) and *env* (envelope proteins) of other retroviruses (Matsukura *et al.* 1987). These extra genes enable HIV to grow more vigorously than other viruses, their exact function not being known.

4.1.1.2 Current Treatment

Preventing AIDS currently resides in avoiding infection from HIV. Several approaches have been taken towards treating HIV infection, amongst them being inhibition of reverse transcriptase, viral assembly or release by drugs or a vaccine. Since 1987 the drug azidothymidine (AZT) has been used to inhibit HIV proliferation, HIV reverse transcriptase using the nucleoside analogue AZT in place of thymidine, AZT incorporation inactivating the HIV DNA. However, AZT is not efficient enough to be a cure, only slowing viral progression (AIDS Research, 1990). The use of antisense ODNs is an alternative approach, selective viral RNA regions being targeted and blocked by hybridisation.

GEM-91, an anti-HIV PS-ODN (Zhang *et al.* 1995), is currently under phase II of clinical trails. This ODN is antisense to the *gag* gene of HIV, showing anti-HIV action *in vitro* (Lisziewicz *et al.* 1994) and relative stability and non-toxicity n human patients (Zhang *et al.* 1995). Anti-HIV ODNs functioning as aptamers, which exert an affect by binding to a protein as opposed to mRNA, have also entered clinical trails (reviewed by Akhtar *et al.* 1997b).

4.1.1.3 Oligonucleotide Therapy for HIV Infection

An array of studies have employed the antisense approach to combat HIV infection and AIDS in the hope of producing a specific, non-toxic remedy (Agrawal *et al.* 1988; Goodchild *et al.* 1988; Matsukura *et al.* 1989; Lisziewicz *et al.* 1994). One of the earliest reports of successful antisense ODN inhibition of HIV replication was by

Matsukura *et al.* (1987 and 1989). Human transformed T-lymphocytes (H9 cells) were chronically infected with HIV-1 and treated with a 27-mer phophorothioate ODN, antisense to the mRNA of the *rev* gene of the virus. The protein product of *rev* is essential for viral reproduction and is known as a regulatory gene, the expression of viral proteins being regulated by this protein (Sodroski *et al.* 1986; Feinberg *et al.* 1986, Jeang *et al.* 1991). Antisense ODN treatment of these infected H9 cells reduced virus production, being attributed to translation arrest of *rev* -encoded protein production (Matsukura *et al.* 1989), the suppressive effects continuing up to 28 days. Zon (1990), Milligan *et al.* (1993), Akhtar *et al.* (1996) and Reddy (1996) review antisense ODN therapy for HIV infection.

4.1.1.4 The *rev* Gene

The *rev* gene is one of the unique genes that HIV possesses, additional to the genes found in other retroviruses (Knight *et al.* 1987). The HIV *rev* protein regulates mRNA transcript splicing/processing, an essential process for viral reproduction (Knight *et al.* 1987). The gene *env*, which encodes the envelope proteins gp120 and gp41, is regulated in part by the *rev* gene. The glycoprotein gp120 is essential for interaction of HIV with its cellular receptor. A down regulation of *rev* expression resulting in inhibition in the production of several viral proteins has been investigated (Matsukura *et al.* 1989; Jeang *et al.* 1991.

4.1.1.5 Antisense Oligonucleotides to the *rev* Sequence

Several studies have used ODN sequences antisense to the start codon of the *rev* gene, with an aim to prevent transcription and *rev* protein production (Matsukura *et al.* 1987 and 1989; Iverson *et al.* 1992; Lisziewicz *et al.* 1993; MacKellar *et al.* 1992). Both studies by Matsukura *et al.* (1987 and 1989) have successfully inhibited several viral proteins in infected cells with the anti-*rev* phosphorothioate ODN, the 1989 study showing an inhibition in overall viral protein production as opposed to just the *rev*

protein. Iverson *et al.* (1992) found nuclear localisation of the anti-*rev* phosphorothioate ODN after 1 hr incubation with cells, four types of cells were found to internalise the ODN, the binding process not appearing to be cell type specific. A phophodiester anti-*rev* ODN was found to be cellularly unstable due to degradation (Matsukura *et al.* 1987) and therefore not an appropriate antisense molecule, viral protein production not being inhibited (Matsukura *et al.* 1989).

4.1.2 ENHANCING THE DELIVERY OF ANTI-HIV OLIGONUCLEOTIDES

Another important factor in ODN design is producing a molecule that will be taken up by cells in a sufficient quantity to reach its target and have an affect. ODNs have been conjugated to various groups or delivered to cells by specific methods in order to enhance binding and uptake. Therefore, with an aim to increasing intracellular bioavailability, the amount of intact ODN available to interact with its target (Budker *et al.* 1992). Rojanaskul (1996) reviews the various measures taken to increase ODN bioavailability, summarising the advantages and disadvantages of such modifications. One of the major advantages of ODN modification is the increase in stability to nuclease degradation, a decrease in affinity of the ODN for the target RNA major disadvantage.

Anti-*rev* ODNs have been encapsulated into antibody targeted liposomes (Zelphati *et al.* 1994) and anti-*tat* ODNs conjugated to poly-lysine (Degols *et al.* 1994). The envelopes of the Sendai virus have been used as a carrier for delivering ODNs to cells. Enhanced delivery of an anti-encephalitis virus ODN to cells was achieved by loading into the envelopes of Sendai virus, an antiviral action resulting (Budker *et al.* 1992). The effect of conjugating lipophilic groups to ODNs is a common strategy employed to enhance ODN cellular association and uptake.

ODNs linked to three different lipophilic groups were evaluated for their cellular association capacity to macrophage cell lines. A cholesterol conjugated ODN

(cholhex), hexa-ethylene glycol conjugated ODN (HEG) and an aliphatic hydrocarbon conjugated ODN (C_{16}) were studied (figure 4.1).

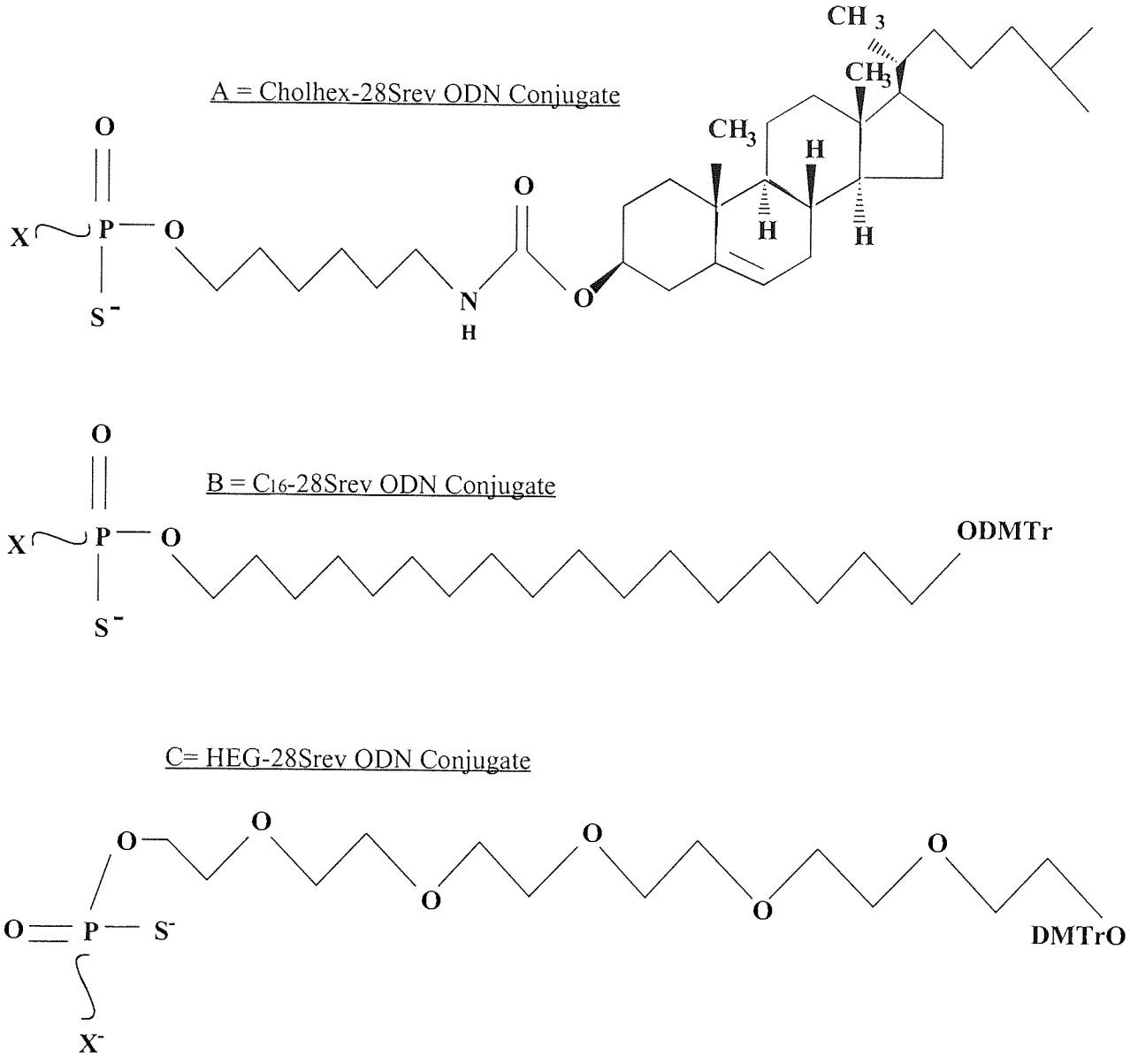


Figure 4.1: Structures of the three 28Srev Oligonucleotide Conjugates, Cholhex (A), C_{16} (B) and HEG (C). 'X' represents the 28Srev ODN, and DMTrO = di-methoxy-trityl, a protecting group (section 2.2.1.1).

4.1.2.1 Cholesterol Oligonucleotide Conjugates

Boutorin *et al.* (1989 and 1992) found a 30-fold increase in cellular association of cholesterol conjugated ODNs, uptake varying between cell lines. Cholesterol has been shown to bind low-density-lipoproteins (LDLs) in plasma, the LDL receptor binds LDLs and internalise the complex into cell by RME. Therefore ODNs conjugated to cholesterol can potentially bind LDL receptors and internalise by RME (Vlassov *et al.* 1994). Kreig *et al.* (1993) report fluorescently labelled cholesterol-conjugated ODNs to enhance ODN uptake and internalise by a RME mechanism as observed by confocal microscopy and flow cytometry. Letsinger *et al.* (1989) show successful inhibition of HIV, in infected cells, by anti-HIV ODNs conjugated to cholesterol. A major disadvantage of cholesterol is its potential to become trapped in the plasma membrane, decreasing bioavailability (Vlassov *et al.* 1994).

There exist a considerable number of studies investigating cholesterol conjugated anti-viral ODNs (Letsinger *et al.* 1989; Stein *et al.* 1991; MacKellar *et al.* 1992; Svinarchuk *et al.* 1993; Demirhan *et al.* 1994). A cholesterol group was conjugated onto an anti-*tat* ODN at the 3'-end in a study by Demirhan *et al.* (1994), this conjugate being found to have a higher efficacy than the unconjugated counterpart to cells transfected with a plasmid containing the *tat* gene, this increased efficacy being attributed to the increase in lipophilicity of the conjugate. Demirhan *et al.* (1994), attribute cell binding and internalisation enhancement of the cholesterol-ODN conjugate to anchoring to lipids in the cell membrane and subsequent endocytosis. Svinarchuk *et al.* (1993) describe the anti-viral effects of a cholesterol conjugated anti-*rev* ODN, the non-cholesterol control ODN being ineffective at inhibiting viral replication.

4.1.2.2 Ethylene-Glycol Oligonucleotide Conjugates

As a means of increasing lipophilicity, ethylene-glycol units are ideal molecules for conjugation to ODNs for a number of reasons, they are non-toxic, non-immunogenic, linear and do not interfere with ODN hybridisation to complementary DNA or RNA

(Jaschke *et al.* 1994; Manoharan *et al.* 1995). Polyethylene glycol (PEG) has been used for protecting proteins from immunogenic response and degradation (Abuchowski *et al.* 1977) and used as a linker between ODNs and membranes (Wang *et al.* 1995). Fuertges *et al.* (1990) found PEG conjugated proteins to have an increased dispersion in water and increased resistance to protease digestion whilst still maintaining activity. The ability of PEG to increase liposome and erythrocyte lipid bilayer permeability is described by Aldwinckle *et al.* (1982). Theoretically PEG conjugation should confer the same characteristics to DNA and ODNs.

The chemistry of ethylene-glycol is such that they can be incorporated into end or internal positions of ODNs by automated synthesis, the size of the polymer can be varied to adjust the hydrophobicity of the resulting conjugate bearing in mind steric hindrance may affect ODN hybridisation to its target (Jaschke *et al.* 1994).

Conjugation of ethylene-glycol units to ODNs increases lipophilicity and exonuclease stability (Jaschke *et al.* 1994), production of the conjugate being relatively easy due to the synthesis of ethylene-glycol phosphoramidites and subsequent solid phase chemistry (Jaschke *et al.* 1994). The increased lipophilicity of ethylene-glycol ODN conjugates predicts a high degree of association with cell membranes compared to the unconjugated ODN. Manoharan *et al.* (1995) however found this not to be the case, cellular association decreasing as a result of ethylene-glycol conjugation. This phenomenon was attributed to ethylene glycol inducing the ODN to bind to extracellular proteins in the biological milieu, therefore decreasing cellular bioavailability (Manoharan *et al.* 1995).

4.1.2.3 Alkyl Chain Oligonucleotide Conjugates

Increasing ODN lipophilicity by conjugation to aliphatic hydrocarbon chains has not been investigated extensively. Kabanov *et al.* (1990), Saison-Behmoaras *et al.* (1992), MacKellar *et al.* (1992) and Temsamani *et al.* (1994) describe the synthesis and use of ODNs linked to an alkyl chain in the aim of increasing ODN lipophilicity, synthesis

being by automated phosphoramidite chemistry. Little information exists regarding ODN-alkyl conjugate cellular association. Kabanov *et al.* (1990) used a conjugate comprising of eleven carbon residues (un-decyl group). The ODN un-decyl conjugate was directed to the Influenza virus in MCDK cells, which caused a significant reduction in synthesis of viral proteins and virus reproduction, non-conjugated ODNs having no affect on viral development.

A phosphorothioate 20-mer ODN linked to a 12-carbon alkyl chain was evaluated by Temsamani *et al.* (1994) for cellular association. The increase in hydrophobicity conferred by the alkyl chain was expected to enhance affinity for the lipophilic cell membranes. This study however revealed that cellular uptake of the ODN was not significantly enhanced by conjugation to the alkyl group, cholesterol conjugation to the same sequence however causing a 4-fold increase in uptake.

4.1.3 ANTISENSE *rev* OLIGONUCLEOTIDE CONJUGATES

Three types of lipophilic moieties, cholesterol (termed cholhex), hexa-ethylene-glycol (termed HEG) and a 16 carbon alkyl chain (termed C_{16}) were conjugated onto an ODN antisense to the *rev* gene of HIV (figure 4.1), all kindly synthesised and provided by Prof. Tom Brown, Dept. of Chemistry, Southampton University (synthesis and physical properties described in MacKellar *et al.* 1992). The conjugation of HEG and C_{16} moieties to ODNs have not been studied extensively, data regarding the efficacy of these ODN conjugates is sparse. A cholhex moiety was conjugated onto an ODN antisense to a region of the cytochrome P450 enzyme mRNA by Desjardins *et al.* (1995), this conjugate significantly reduced activity of the enzyme compared to an unconjugated control ODN. The antisense *rev* ODN sequence, section 4.2.2, has been used in many studies to inhibit HIV infection (section 4.1.1.5).

4.1.4 AIMS - *rev* OLIGONUCLEOTIDE CONJUGATES

The effects of conjugating these lipophilic moieties to ODNs, in terms of stability to degradation and cellular association, was assessed in this chapter. These conjugates were assessed for cellular association in two cell lines, RAW264.7 and A431, their general binding and uptake characteristics were compared to an unconjugated control ODN. The cellular association of various homo-ODNs was also assessed in a murine macrophage and human epithelial cell line.

4.2 MATERIALS AND METHODS

4.2.1 LIPOPHILIC 28-mer *rev* OLIGONUCLEOTIDE CONJUGATE SYNTHESIS

The conjugates used in this thesis (figure 4.1) were a generous gift from Prof. Tom Brown (Southampton University). The synthesis, purification and identification of the cholhex, C_{16} and HEG 28-mer *rev* ODN conjugates is described by MacKellar *et al.* (1992).

4.2.2 UNCONJUGATED 28-mer *rev* OLIGONUCLEOTIDE SYNTHESIS

All unconjugated ODNs (28Srev and 28Drev) were synthesised and prepared for use according the method described in chapter 2, section 2.2.2, by an automated DNA/RNA synthesiser. The ODNs were designed to be complementary to the region from the 2nd to the 29th bases of the *rev* gene of the HIV-1. All *rev* conjugated and unconjugated ODNs sharing this common 28-mer sequence:-

28mer *rev* antisense sequence

$^{5'}$ TCG TCG CTG TCT CCG CTT CTT CCT GCC A$^{3'}$

The 28mer *rev* nonsense sequence was designed by the SERC Daresbury Seqnet program (accessed via telnet), conserving the base ratio and structural energy of the antisense sequence:-

$^{5'}$ CTC ACT CTG CTC TCT CGT CGT TCC GCT G$^{3'}$

4.2.3 THE DETERMINATION OF OLIGONUCLEOTIDE LIPOPHILICITY

The unconjugated 28Srev ODN and cholhex, C_{16} and HEG ODN conjugates were assessed for relative lipophilicity by the method described by Dagel *et al.* (1991) and Beck *et al.* (1996). The ODN were 3'-end ^{32}P-labelled (section 2.3.1.1) and a quantity amounting to 10 000 counts (hand held Geiger counter) was dried under vacuum in six replicate 1.5ml screw-top microfuge tubes. The ODN residue was resuspended in 500µl of ddH_2O (aqueous phase) and 500µl of octanol (organic phase) (Sigma-Aldrich, Poole, U.K.) added. The mixture was shaken for 3hours at room temperature and allowed to settle overnight to separate the two phases. 300µl of the octanol phase was carefully removed and placed into a scintillation vial containing 10ml of scintillation fluid as was 300µl of the ddH_2O phase which was placed into a separate scintillation vial. The vials were assessed for radioactivity associated with each phase as described in section 2.3.3.

The partition coefficient (log P) was expressed as log of the amount of ODN present in the organic phase compared to the amount present in the aqueous phase:-

$$\text{Log P} = \log \frac{[\text{ODN}]_{\text{octanol}}}{[\text{ODN}]_{\text{ddH2O}}}$$

4.2.4 SOUTH-WESTERN BLOTTING

A modifed version of the method used by Bennett *et al.* (1985) and Akhtar *et al.* (1996) to isolate DNA or ODN binding proteins, was used and discussed below.

4.2.4.1 The Isolation of RAW264.7 Cell Membranes

RAW264.7 cell membranes were prepared by adaptation of a method described by Dr. J. Coulson (personnal communication, Aston University). The macrophages, 5 x 10^5

per well, were cultured in 24 well-tissue culture plates as described in section 2.1.5, triplicate wells washed three times with 0.5ml PBS, ensuring all traces of serum containing media was removed, and solubilised with 250µl/well harvesting solution (0.05M boric acid, 0.15M NaCl, 1mM $MgCl_2$, pH7.2). Cells were scraped (using a 1ml pipette tip with its end covered with Parafilm) to dislodge them from the well and collected in 1.5ml microfuge tubes, centrifuging at 450rpm for 5 minutes. The pellet formed was resuspended in harvesting solution (100µl) and added slowly to 5ml extraction solution (0.02M boric acid, 0.2mM EDTA, pH10.2) whilst stirring, mixing for a further 10 minutes. Borate solution (0.5M boric acid pH 10.2) was added (400µl) and the resulting solution filtered through a small cotton wool plug and centrifuged at 450rpm for 10 minutes at 2°C (to sediment cell debris), the supernatant being collected and recentrifuged for 30 minutes at 12 000rpm (2°C). The pellet was resuspended in 5ml PBS and layered onto 5ml sucrose (35% in PBS), centrifuging at 17 500rpm for 1 hour at 2°C. Membranes were collected from the interface of the resulting centrifuged solution with a Pasteur pipette, a pellet being collected after centrifugation at 36000rpm for 10 minutes at 2°C and stored at -20°C.

4.2.4.2 Membrane Protein PAGE

Mini gel electrophoresis plates were assembled as directed by the manufacturer (Bio-Rad, Hertfordshire, U.K.). A polyacrylamide running gel mixture (approximately 10ml) was prepared by mixing 3.3ml distilled water, 4.0ml of 30% acrylamide/bis-acrylamide mixture (Severn Biotech, Worcester, U.K.), 2.5ml of 1.5M tris (pH8.8), 0.1ml of 10% $^{w}/_{v}$ sodium dodecyl sulphate (SDS), 0.1ml of 10% $^{w}/_{v}$ ammonium persulphate (AMPS) and 4µl TEMED (all ICN Biomedicals, Oxfordshire, U.K.) and allowed to polymersise for 30-35 minutes at room temperature, before stacking gel (approximately 3ml) was added comprising of 2.1ml distilled water, 0.5ml of 30% acrylamide/bis-acrylamide mixture, 0.38ml of 1.0M tris (pH6.8), 30µl of 10% SDS, 30µl of 10% AMPS and 3µl TEMED, a well-forming comb was placed between the plates and allowed to polymerise for 30-35 minutes at room temperature. Following polymerisation, the comb was removed and the wells washed with distilled water, the

gel plates with gel were assembled into the electrophoresis apparatus containing running buffer (24mM tris base, 192mM glycine, 1% SDS, all ICN Biomedicals, U.K.).

Each membrane preparation aliquot (section 4.2.4.2) was separated into four parts and each loaded with 10µl standard dissociation buffer (10x) comprising of 0.1M tris, 1mM EDTA, 1% SDS and 1ml mercaptoethanol (pH 7.2) into a separate well of a mini SDS-polyacrylamide gel (section 2.3.2.3), alongside MW markers (205kDa-29kDa range, Sigma-Aldrich, Poole, U.K.), electrophoresing at 60volts for 1 hour in running buffer. The resulting gel was washed in distilled water and soaked in 10ml 0.1% Coomassie solution (Sigma, Poole, UK), used to stain the separated proteins. After recording the positions of the proteins with respect to the MW markers the gel was washed in distilled water several times to remove the Coomassie stain and washed twice in freshly prepared transfer buffer, comprising of 25mM Tris, 192mM glycine and 20% $^{v}/_{v}$ methanol (ICN Biomedicals, UK).

4.2.4.3 South Western Transfer and Blotting

RAW264.7 cell membrane proteins separated on the gel were electrophoretically transferred to a nitrocellulose filter by Western transfer. The transfer sandwich (Bio-Rad Transfer System, Hertfordshire, UK) was assembled with a piece of pre-wet (with transfer buffer) nitrocellulose filter (Millipore, Bedford, UK) cut to the size of the gel, and six pieces of pre-wet filter paper (Whatman, UK) following the protocol outlined by the manufacturers. The transfer tank was connected to the power source and programmed to transfer for 2 hours at 60 volts.

Following transfer, both the nitro-cellulose and gel were washed in distilled water twice, the nitrocellulose filter was soaked in Ponceau-S for 10 minutes and the gel in 0.1% Coomassie blue (Sigma-Aldrich, Poole, UK), in order to detect and record the positions of proteins and observe the efficiency of transfer, following which the Ponceau-S was washed off with distilled water.

4.2.4.4 Oligonucleotides Incubation and Autoradiography

Srev ODNs (28Srev, cholhex, C16 and HEG) were 3'-end ^{32}P-ATP labelled (section 2.3.1.1) and each incubated with a piece of nitrocellulose filter containing the transferred proteins. The ODN (approximately 1000 counts on the Geiger counter) and nitrocellulose filter were sealed in a bag (ensuring no air bubbles) with standard binding buffer (approximate volume 5ml) composed of 10mM Tris-HCl (pH7.0), 0.5% gelatin, 0.02% ficoll, 1mM EDTA, 0.05% NaCl and 1% Triton-X, and rolled slowly in a large tube overnight at room temperature.

Following incubation the nitrocellulose filter was washed five times with distilled water to remove excess ODN and binding buffer. A piece of autoradiography film was placed either side of the nitrocellulose filter or exposed for seven days in the dark, after which the film was developed (section 2.3.2.3).

4.2.5 CELLULAR ASSOCIATION OF HOMO-OLIGONUCLEOTIDES

4.2.5.1 Homo-Oligonucleotides

Homo-ODNs were synthesised as described in section 2.2.2, the various sequences used listed below:-

ODN Chemistry	Length and Sequence
Homo-Thymidine (PS)	7T, 14T, 20T, 28T, 36T e.g. 7T:- $^{5'}$TTT TTT T $^{3'}$ 14T:- $^{5'}$TTT TTT TTT TTT TT $^{3'}$
Homo-Cytidine (PS)	7C:- $^{5'}$CCC CCC C $^{3'}$
Homo-Adenine (PS)	7A:- $^{5'}$AAA AAA A $^{3'}$
Homo-Guanine (PS)	7G:- $^{5'}$GGG GGG G $^{3'}$

4.2.5.2 The Effect of Base Composition

The cellular association of various phosphorothioate homo-ODNs, 7A, 7,G, 7T and 7C (section 4.2.5.1), was assessed in both RAW264.7 and A431 cells after 60 minutes incubation at 37°C and 7A and 7G homo-ODNs were also assessed for cellular association at 4°C to these cell lines, protocol described in section 2.5.2.

4.2.5.3 The Effect of Oligonucleotide Length

Phosphorothioate homo-thymidine ODNs of different length, 7T, 14T, 20T, 28T and 36T (section 4.2.5.1) were 5'-end labelled and assessed for cellular association in RAW264.7 and A431 cells at 37°C after 60 minutes incubation, protocol described in section 2.5.2.

4.3 RESULTS

4.3.1 OLIGONUCLEOTIDE LIPOPHILICITY

The cell membrane acts as a selective barrier to exogenous material. Conjugation of lipophilic moieties to ODNs is performed to enhance cellular uptake across the plasma membrane, native ODNs being polyanionic in nature and thus not permeable to the lipid membrane (Budker *et al.* 1992).

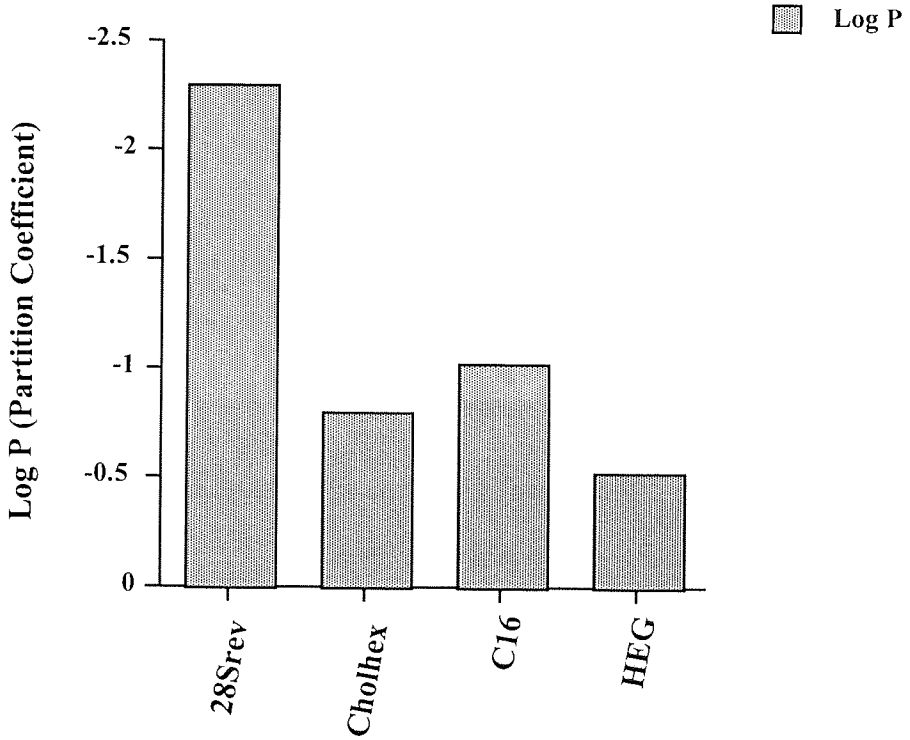
Studies, which describe the synthesis of the cholhex, C16 and HEG ODN conjugates (MacKellar et al. 1992), have stated that they are more lipophilic than corresponding unconjugated ODNs. Beck *et al.* (1996) and Dagel *et al.* (1991) describe a method by which the relative lipophilicities of ODNs can be assessed by monitoring partitioning between an aqueous and organic phase, where the organic phase represents the biological membrane (section 4.2.3).

Shown in figure 4.1 are the structures of the lipophilic moieties conjugated to the 28Srev ODNs; cholhex (4.1A), C_{16} (4.1B), and HEG (4.1C). All four of these Srev ODNs were assessed for relative lipophilicity by monitoring partitioning between a water and octanol mixture using the method described by Dagel *et al.* (1991). The partition coefficient represents the ratio of ODN found in each phase, being expressed as the log of this amount, logP (section 4.2.3).

An increase in lipophilicity will cause the molecule to partition into the organic phase (octanol). The conjugate moieties, cholhex, C_{16} and HEG, influences the overall negative charge of the ODN *per se* making the molecule more hydrophobic (Dagle *et al.* 1991). The partitioning of the Srev ODNs between the water/octanol mixture is

shown in figure 4.2. Higher lipophilicity is indicated by a low logP value, a low value represents a greater partitioning into the organic phase (section 4.2.3).

Figure 4.2 : Lipophilicity of the 28Srev Oligonucleotide and Conjugates. Graph to show the effects of various lipophilic groups on the lipophilicity of the 28Srev oligonucleotides. Oligonucleotides were 3'-end labelled, shaken in equal volumes of octanol and water and the radioactivity in each phase assessed. Values represent log of the ratio of oligonucleotide in the octanol phase comparedto water. n=5.



All three conjugated Srev ODNs were more lipophilic than the unconjugated control Srev, as predicted (figure 4.2). HEG was found to be most lipophilic of all three conjugates, being 4-fold more lipophilic than the control 28Srev ODN. The C_{16} conjugate was the least lipophilic, having a logP approximately 2-fold lower than the control 28Srev ODN.

4.3.2 CELLULAR ASSOCIATION OF 28Srev OLIGONUCLEOTIDES

The cellular association of the mono-mannose conjugate ODN, in murine macrophage cell lines, was discussed in chapter 3.0 and the effects of various exogenous factors evaluated. Similar experiments were applied to the 28Srev ODN and its lipophilic conjugates, their binding characteristics were also studied. Each of the three lipophilic conjugates were studied for efficacy of cellular association, comparisons made to the unconjugated control 28Srev ODN.

RAW264.7 cells were used as a model cell line; some studies were performed in the epithelial cell line A431 (human squamous carcinoma) for comparison. Macrophages are a natural harbour for HIV, therefore an ideal target model cell line for antisense *rev* ODNs. Trypan blue exclusion tests did not reveal any toxicity, to either cell line, during any of the experimental incubations or treatments described in sections 4.3.2.1 to 4.3.2.13 (data not shown).

4.3.2.1 Stability of the Srev Oligonucleotide and Conjugates in Cell Culture

The stability of the 28Srev ODN and cholhex, C_{16} and HEG conjugates was evaluated in RAW264.7 cells cultured in serum free D-MEM, as described in section 2.4. Therefore ODN degradation resulting from nucleases released from the cells could be assessed.

Shown in figures 4.3 and 4.4 are the stability profiles for the Srev ODNs when incubated with RAW264.7 cells for different incubation periods. The cellular nucleases released by these macrophages into the medium degraded the various 28Srev ODNs to different degrees, degradation resulted in shorter ODN strands which appear as separate bands following electrophoresis.

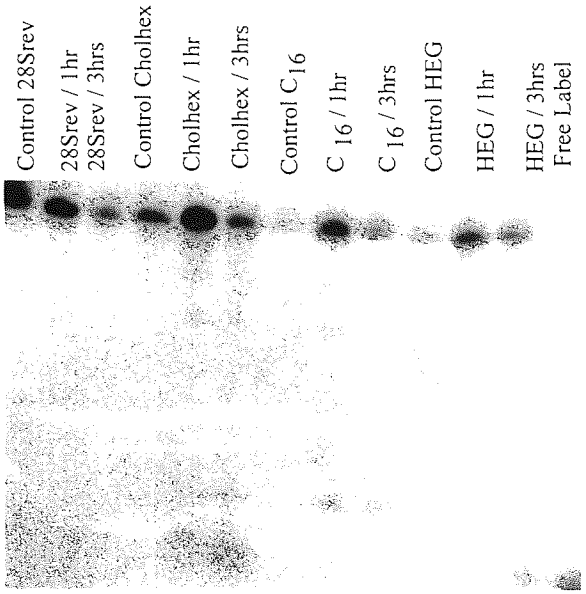


Figure 4.3: Stability of the Srev Oligonucleotide and Conjugates following 1 and 3 hrs incubation at 37°C, with RAW264.7 Cells. Autoradiograph of 3'-end ^{32}P-labelled ODNs electrophoresed by 20% PAGE. ^{32}P-labelled ODNs electrophoresed without prior incubation with cells were designated 'controls'.

None of the ODNs showed degradation after 1hr and 3hrs incubation with the RAW264.7 cells (figure 4.3). Therefore the 28Srev ODN and conjugates, incubated with RAW264.7 cells for up to 3hrs, could be assumed as intact and full length.

Experiments involving endocytosis inhibitors (sections 4.3.2.12 and 4.3.2.13) required the 28Srev ODNs to be incubated with cells for 4 hours at 37°C. Therefore ODNs were assessed for stability in cells over a longer time period of 5 hrs.

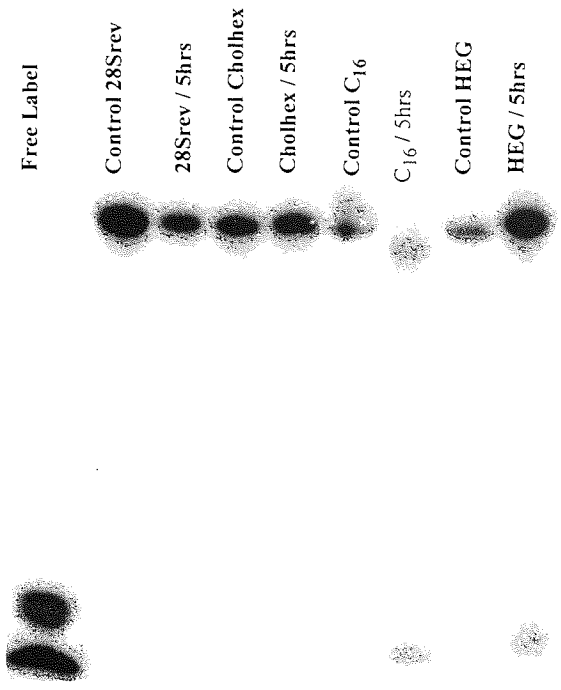


Figure 4.4: Stability of the Srev Oligonucleotide and Conjugates following 5 hrs incubation at 37°C, with RAW264.7 Cells. Autoradiograph of 3'-end ^{32}P-labelled ODNs electrophoresed by 20% PAGE. 28Srev ODN.

None of the ODNs showed degradation after 1hr and 3hrs incubation with the RAW264.7 cells (figure 4.3). Therefore the 28Srev ODN and conjugates, incubated with RAW264.7 cells for up to 3hrs, could be assumed as intact and full length.

Experiments involving endocytosis inhibitors (sections 4.3.2.12 and 4.3.2.13) required the 28Srev ODNs to be incubated with cells for 4 hours at 37°C. Therefore ODNs were assessed for stability in cells over a longer time period of 5 hrs.

The stability profile for the 28Srev ODN and conjugates incubated with RAW264.7 cells for 5hrs is shown in figure 4.4. Free ^{32}P-ATP bands were detected in 28Srev ODN, cholhex, C_{16} and HEG conjugates incubated with RAW264.7 cells indicating degradation. The C_{16} conjugate was most unstable of all the ODNs, virtually all intact ODN degraded to shorter fragments, seen as a laddering pattern (figure 4.4).

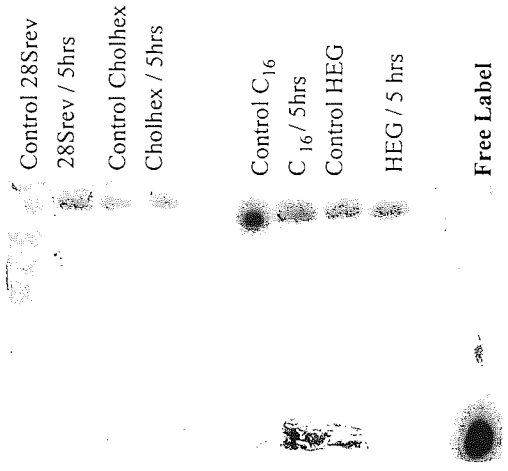


Figure 4.5: Stability of the Srev Oligonucleotide and Conjugates following 5 hrs incubation at 37°C, with A431 Cells. Autoradiograph of 3'-end ^{32}P-labelled ODNs electrophoresed by 20% PAGE.

Stability of the ODNs was also assessed with A431 cells after an incubation period of 5 hours at 37°C (figure 4.5). No degradation was observed on incubation with A431 cells. A431 cells appear to release a lower quantity of degradative nucleases, compared to the RAW264.7 macrophages, highlighting cell-cell variation.

Following 5hrs incubation at 37°C with macrophages, any information obtained regarding cellular association, particularly for the C_{16} conjugate, could be open to mis-interpretation. Values for associated radioactivity at this time period could partly be due to free ^{32}P-ATP and shorter ODN fragments. Therefore subsequent studies involving co-incubation of cells with these ODNs were not performed for periods longer than 3 hours, other than the study involving the endocytosis inhibitors (sections 4.3.2.12 and 4.3.2.13). We can conclude from these stability assays that, aside from the C_{16} conjugate, there was no significant degradation in the medium of either RAW264.7 or A431 cells of the various Srev ODNs when incubated for up to 5 hours at 37°C.

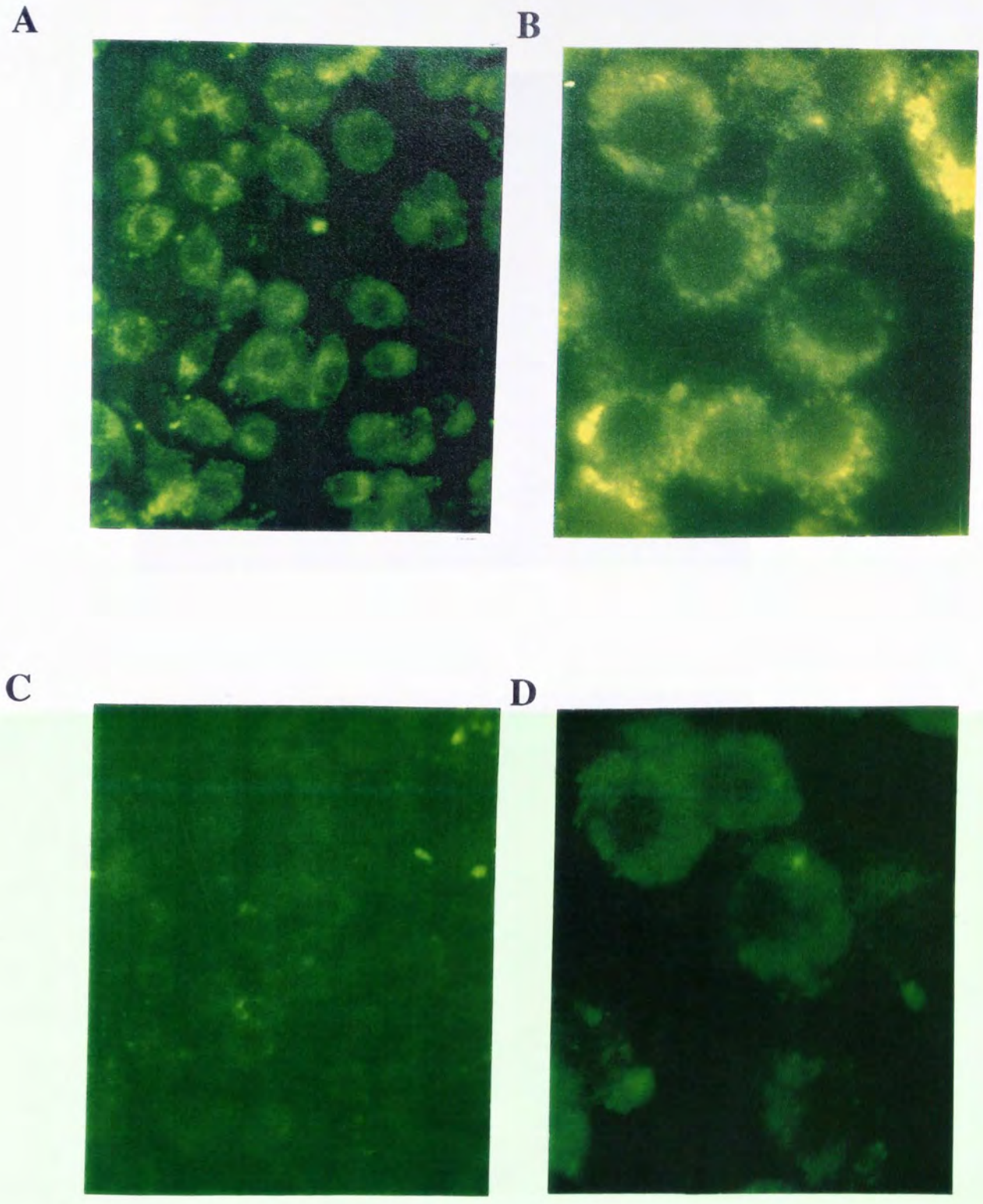
4.3.2.2 The Binding of Fluorescein-Oligonucleotide-Cholesterol Conjugates

Many studies use fluorescent analysis, in the form of microscopy or fluorescent activated cell sorting (FACS), as a means of assessing the fate of ODNs within cells. Fluorescently labelled cholesterol Srev ODNs, of identical backbone and sequence were not available, however, a chimeric cholesterol conjugated ODN was tested, a generous gift from Dr. D. Spiller (Liverpool University). In order to gain information about the cellular association characteristics and intracellular fate of cholesterol conjugated ODNs to RAW264.7 cells, this conjugate was investigated. Fluorescent microscopy may shed light not only on any enhanced cellular association, which the cholesterol moiety conveyed on the ODN, but also on the mechanism by which binding and internalisation was occurring.

This fluorescently labelled ODN differed from the cholhex conjugate in terms of size, backbone and type of label, therefore it was not possible to make direct parallels between the binding and distribution of this and the cholhex conjugate. The fluorescent ODN was an 18-mer with a chimeric backbone, the four flanking bonds either end of the molecule were methylphosphonates and the middle nine bonds were phosphodiesters. The fluorescent label was also on the 3'-end of the cholesterol conjugate and the 5'-end of the control. The differing nature of the cholhex and fluorescent-cholesterol ODN probably has a great bearing on cellular association. However, the influence of the cholesterol moiety on cellular association and distribution in RAW264.7 cells could be assessed, regardless of ODN nature.

RAW264.7 cells were incubated with the cholesterol conjugated chimeric and control ODNs for 60 minutes prior to photography (section 2.6). Figures 4.6A and 4.6B show the cholesterol conjugated chimeric ODN and figures 4.6C and 4.6D the control unconjugated chimeric ODN. The cells were also photographed under phase contrast after incubation with cholesterol conjugated chimeric ODN (figure 4.7A) and control unconjugated chimeric ODN (figure 4.7B), to highlight the fact that cell density was equal, any difference in fluorescence intensity attributable to fluorescent ODN binding only.

The fluorescent micrographs clearly show a difference on fluorescence intensity between the cholesterol-conjugated and unconjugated ODNs (figures 4.6A and B, and 4.7A and B). A punctate distribution of fluorescence in the cells incubated with the cholesterol conjugated ODN was observed (figures 4.6A and 4.7A), which is characteristic of cells internalising material into intracellular vesicles as opposed to a dispersed random distribution in the cytoplasm (Bennett *et al.* 1992; Noonberg *et al.* 1993; Shoji *et al.* 1996). The central nuclear region remained relatively free from fluorescence, indicating that there was no or little uptake into the nucleus. Intracellular fluorescent vesicular distribution was mainly around the periphery, particularly apparent in photographs taken under a higher magnification (figure 4.6A). Boutorine *et al.* (1993) found 80% of the total bound fraction of a cholesterol conjugated ODN on the surface of bladder carcinoma cells, the majority of the remainder in the cytoplasm, fluorescent analysis showing small fluorescent endosome-like particles within the cell and a little nuclear localisation.



Figures 4.6A, B, C and D: Fluorescent Micrographs of RAW264.7 Cells. 1.25 x 10^5/ml cells seeded per chamber 24 hours prior to 4hrs incubation with: (A and B) cholesterol conjugated chimeric ODN, (C and D) unconjugated chimeric ODN at 37°C, following which cells were washed and visualised by a Jenamed fluorescence microscope (A and C at 50x, and B and D at 100x magnification).

A

B

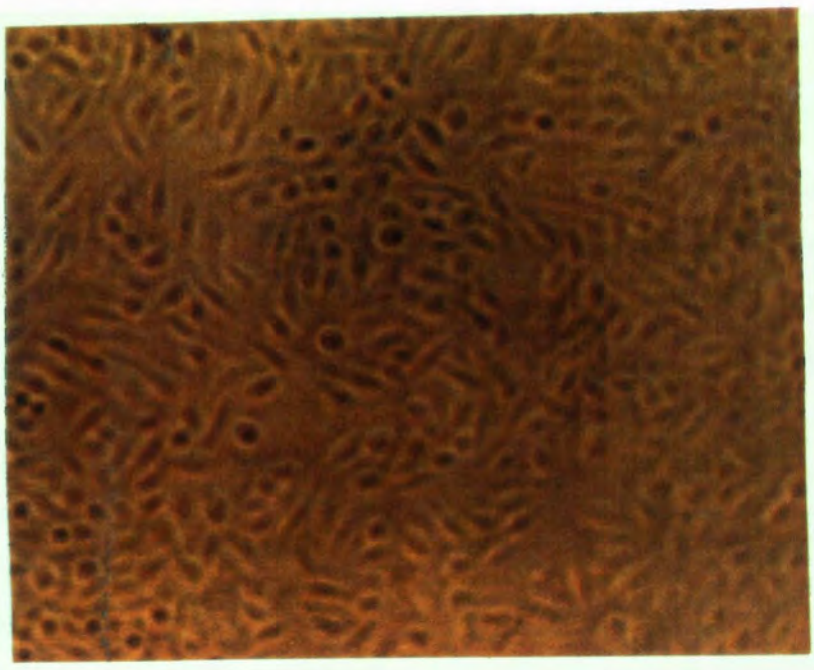


Figure 4.7 (A and B). Phase Contrast of RAW264.7 Cells. 1.25 x 10^5/ml RAW264.7 cells seeded per well of a chamber slide 24 hours prior to incubation with the fluorescein labelled chimeric ODN for 4hrs at 37°C, following which cells were washed to remove ODN traces and visualised under phase contrast with a Jenamed fluorescence microscope under 50x magnification. Cells incubated with (A) Cholesterol conjugated chimeric ODN, (B) Unconjugated control chimeric ODN.

The control unconjugated ODN also bound RAW264.7 cells, again on the periphery, the nuclear region remaining relatively clear of fluorescence (figures 4.6B and 4.7B). However the same punctate pattern was less apparent, with the distribution appearing more uniform. A lower overall fluorescence intensity also indicated a lower degree of control fluorescent-ODN binding. Kreig *et al.* (1993) report enhanced internalisation of a cholesterol conjugated ODN, as assessed by confocal microscopy, in comparison to an unconjugated control ODN.

As stated earlier, these data are not directly comparable to cholhex conjugate binding and distribution in RAW264.7 cells but confirms that a cholesterol moiety on ODNs enhances binding to the cells. The punctate pattern resembled that of a material internalised by endocytosis involving intracellular vesicles. Whether internalisation is via a passive pinocytosis or a receptor mediated mechanism cannot be determined by this data alone.

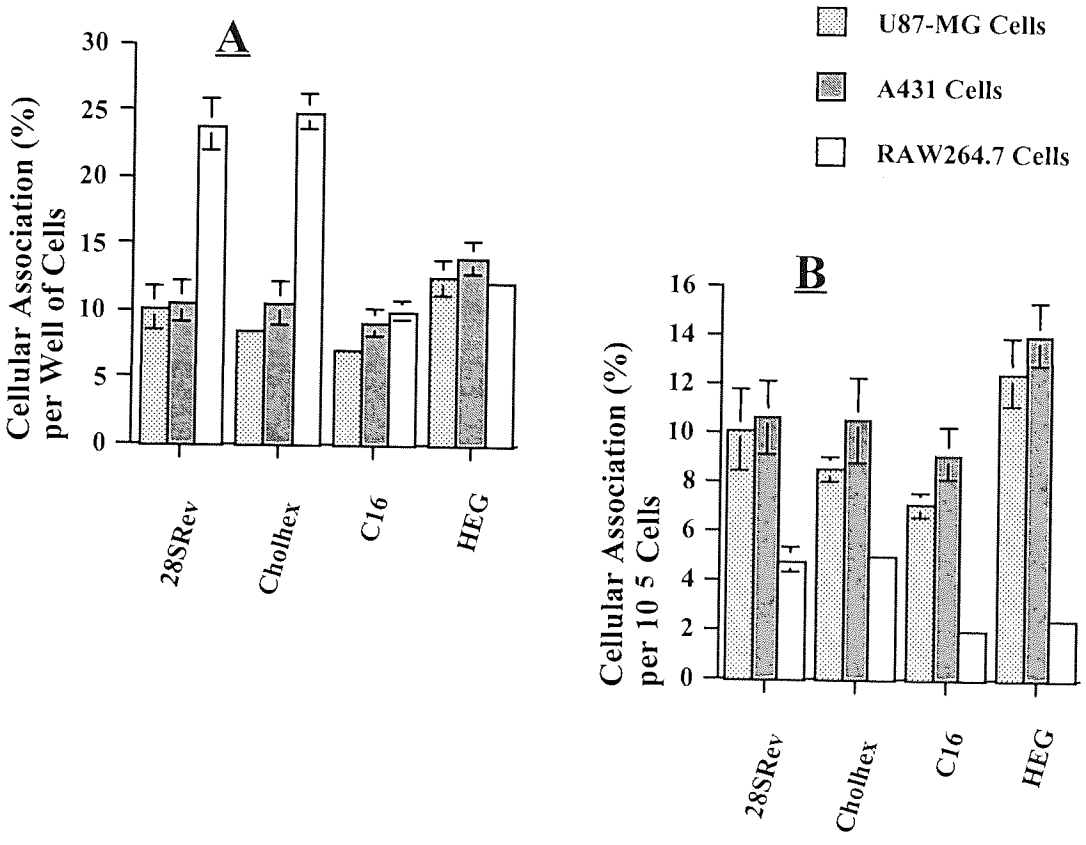
4.3.2.3 Comparison of Cellular Association in Different Cell Lines

ODN binding and uptake characteristics *in vitro* vary considerably between different cell lines (Noonberg *et al.* 1993; Temsamani *et al.* 1994: Beck *et al.* 1996). Cell line characteristics are equally as important as those of the ODN in determination of the relative binding potential. The plasma membrane is an important cell boundary involved in trafficking of molecules in and out of cells, therefore cell membrane composition, putative surface binding molecules, cell size, morphology and *in vivo* functions all contribute to the different ODN binding.

In order to gain a cellular association profile for the Srev ODNs, three cell lines, murine RAW264.7 macrophages, human A431 vulval epithelial cells and U87-MG human glioma cells (described in section 2.5.2), were used to assay binding of these ODNs. Cellular association data was calculated as the percentage association per well of cells (figure 4.8A) and per 10^5 cells (figure 4.8B). Time constraints did not permit a broad range of cell types to be investigated. However, each of the selected cell lines

differ in their biological function, their expression levels of membrane proteins and surface binding molecules. Therefore, binding of the Srev ODNs in this panel of cell lines should represent general trends in binding characteristics and demonstrate any enhancement in binding conferred by the lipophilic modifications.

Figures 4.8 (A and B) : Cellular Association of the Srev and Conjugate ODNs with Cultured Cells. Graphs to show the cellular association (%) of the Srev ODNs with different cell lines (RAW264.7, A431 and U87) following 1hr incubation at 37oC, % expressed per well of cells (A) or per 10^5 cells (B). n=4, bars=SD



The 28Srev ODN and cholhex conjugate bound to RAW264.7 cells to a significantly higher degree than the other two cell lines, when calculated per well (figure 4.8A). When cellular association of the Srev ODNs was calculated as a percentage per 10^5 cells a very different profile was seen. RAW264.7 cells had the lowest cellular associations of the 28Srev and cholhex ODNs, approximately 50% that for the A431

and U87-MG cells and the C_{16} and HEG conjugates bound to approximately 25% and 20% that of A431 and U87-MG cells respectively.

Neither the C_{16} or HEG conjugate moieties appear to enhance cellular association of the 28Srev sequence in these three cell lines. This data indicates that the association of the C_{16} and HEG conjugates may be cell line independent, as similar levels of cellular association were seen in all three cell lines. However, a greater number of cell lines must be investigated before any generalisations can be made as to ODN binding characteristics.

4.3.2.4 Comparison of the Cellular Association of Antisense and Nonsense 28Srev Oligonucleotides

Cellular association of the unconjugated 28Srev ODN was higher than the C_{16} and HEG conjugates with the RAW264.7 cells. This is contrary to the predicted outcome, as the lipophilicity of these conjugates was expected to convey a higher level of cellular association.

Phosphorothioates generally demonstrate more avid cellular association then phosphodiesters, which is generally attributed to binding of surface proteins as a result of their strong anionic nature. However, this *rev* sequence had an unusually high cellular association, approximately 20% to 25% of the total amount of the 28Srev ODN associated with the cells after 60 minutes incubation at 37°C. Iverson *et al.* (1992) reported <0.1% cellular association for a 27Srev ODN in a hamster lung cell line after 60 minutes incubation at 37°C. This ODN differed by only 3 codons from the 28Srev ODN described in this chapter. Therefore, there is a 250-fold difference in magnitude of cellular association between these sequences, which could be a function of experimental conditions, ODN length, cell line or various other factors (pH, temperature and cell passage number).

Approximately 12% to 16% of C_{16} and HEG conjugates bound to RAW264.7 cells respectively under identical conditions. Therefore it appears that the C_{16} and HEG moieties hinder association of the ODN to RAW264.7 cells.

A possible explanation for such high cellular association could be that the 28Srev sequence folds to form a secondary structure. This secondary structure may have spatial arrangement, such as folding into a more compact structure, which enhances the ODNs ability to associate with and possibly internalise into RAW264.7 cells efficiently. If this were the case then the C_{16} and HEG moieties may interfere with the formation of such a structure, impeding their cellular association. The cholhex conjugate showed a higher degree of cellular association compared to the unconjugated 28Srev ODN, probably attributable to the cholesterol moiety (Kreig *et al.* 1993).

To determine whether the particular antisense 28rev sequence was influencing cellular association a 28-mer nonsense sequence was designed, the antisense 28rev base ratio being maintained, but the sequence scrambled. A number of 28Srev-nonsense sequences were analysed by the 'SERC Daresbury RNA-fold' program (section 4.2.2), to predicts the probability of secondary structure formation. A nonsense sequence, with a folding energy (data no shown) similar to the antisense 28Srev sequence, was synthesised and used for comparison of cellular association to the antisense 28Srev ODN (figure 4.9).

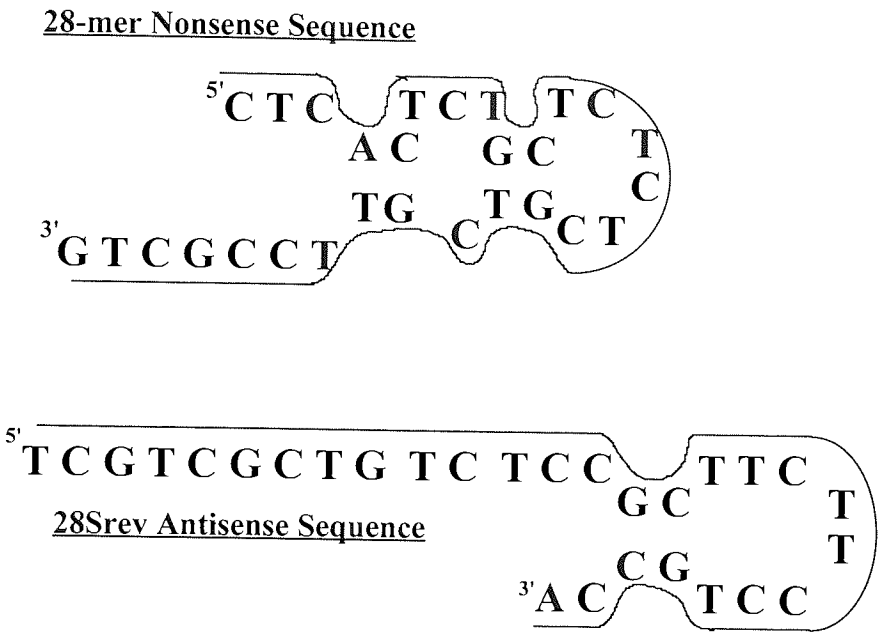
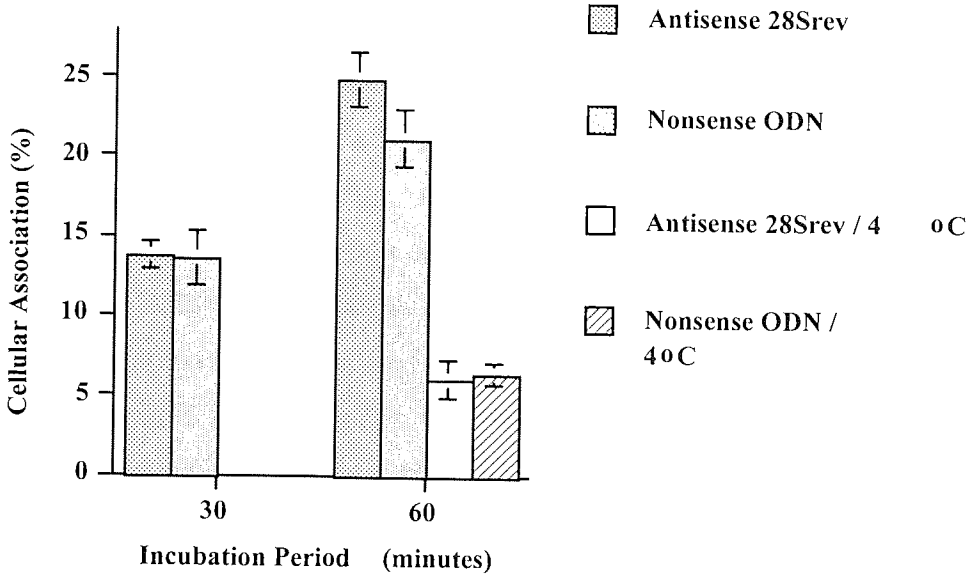


Figure 4.9: Schematic Representation of Nonsense and Antisense Sequences. Folding predicted by the SERC-Daresbury RNA-fold program.

Cellular association of 28Srev antisense and nonsense sequences (section 4.2.2) were assessed in RAW264.7 cells at 30 minutes and 60 minutes at 37°C, and 60 minutes at 4°C in the usual way, (figure 4.10)

As shown in figure 4.10, there was no statistical difference between cellular association of antisense and nonsense sequences after 30 minutes incubation at 37°C ($p=0.46$), however, after 60 minutes incubation cellular association did not differ significantly ($p=0.15$), the antisense sequence binding to a slightly higher degree. Both sequences bound to similar degrees after 60 minutes incubation at 4°C ($p=0.3$). The antisense sequence had a 3.5% increased cellular association compared to the scrambled nonsense ODN.

Figure 4.10 : Cellular Association of Antisense 28Srev and Nonsense ODNs with RAW264.7 Cells. Graph to show the cellular association (%) of an antisense 28Srev ODN comparedwith a nonsense ODN sequence with RAW264.7 cells, 1 hr incubation at 37°C and 4°C. n=4, bars=SD



Closer inspection of the nonsense sequence revealed that both this and the antisense sequence shared a short four base consensus sequence, $^{5'}$ CCGC $^{3'}$, although the antisense sequence was randomly scrambled. This particular sequence may be the cause of such high cellular association of both sequences. Four 'G' base quartets are known to cause increased cellular association of ODNs (Hughes *et al.* 1994; Peyman *et al.* 1995; Lederman *et al.* 1996). An intra-strand G-tetrad can potentially be formed when four-G bases are found consecutively in a sequence in the presence of divalent cations (Na^+ or K^+). The same may also be true of $^{5'}$CCGC$^{3'}$, found in both the antisense and nonsense Srev ODN, an intra-strand structure forming which enhances cellular association.

4.3.2.5 Cellular Association of Srev Oligonucleotides and Fluid Phase Markers over Time, and Effects of Temperature

If the Srev ODNs associate with cells via a mechanism involving specific binding proteins, which enable internalisation into the cell, then it is likely that energy is required for this process. As described in section 2.5.2.3, cellular energy is produced by the action of enzymes, which function under specific optimal conditions such as physiological temperature. In order to gain information about the mechanism involved in binding and uptake the cellular association of 28Srev, 28Drev and conjugates was evaluated at 37°C and 4 °C over a range of incubation periods (30, 60, 180 and 300 minutes) in RAW264.7 cell (section 2.5.2.3). The cellular association of a fluid phase marker, ^{3}H-mannitol, was compared with the ODNs, differences in the profiles gave an indication of different mechanisms. The use of fluid phase markers and some of their characteristics are described fully in section 2.5.2.2. The profiles for cellular association at 37°C and 4°C are shown in figures 4.11A (28Srev compared to 28Drev) , 4.11B (28Srev compared to cholhex), 4.11C (28Srev compared to C_{16}) and 4.11D (28Srev compared to HEG).

A significant difference is seen between the binding of phosphodiester (28Drev) and phosphorothioate (28Srev) backbones in RAW264.7 cells (figure 4.11A). This was the predicted outcome, phosphorothioate ODNs bind to cell surface proteins more readily due to the sulphur groups in the backbone.

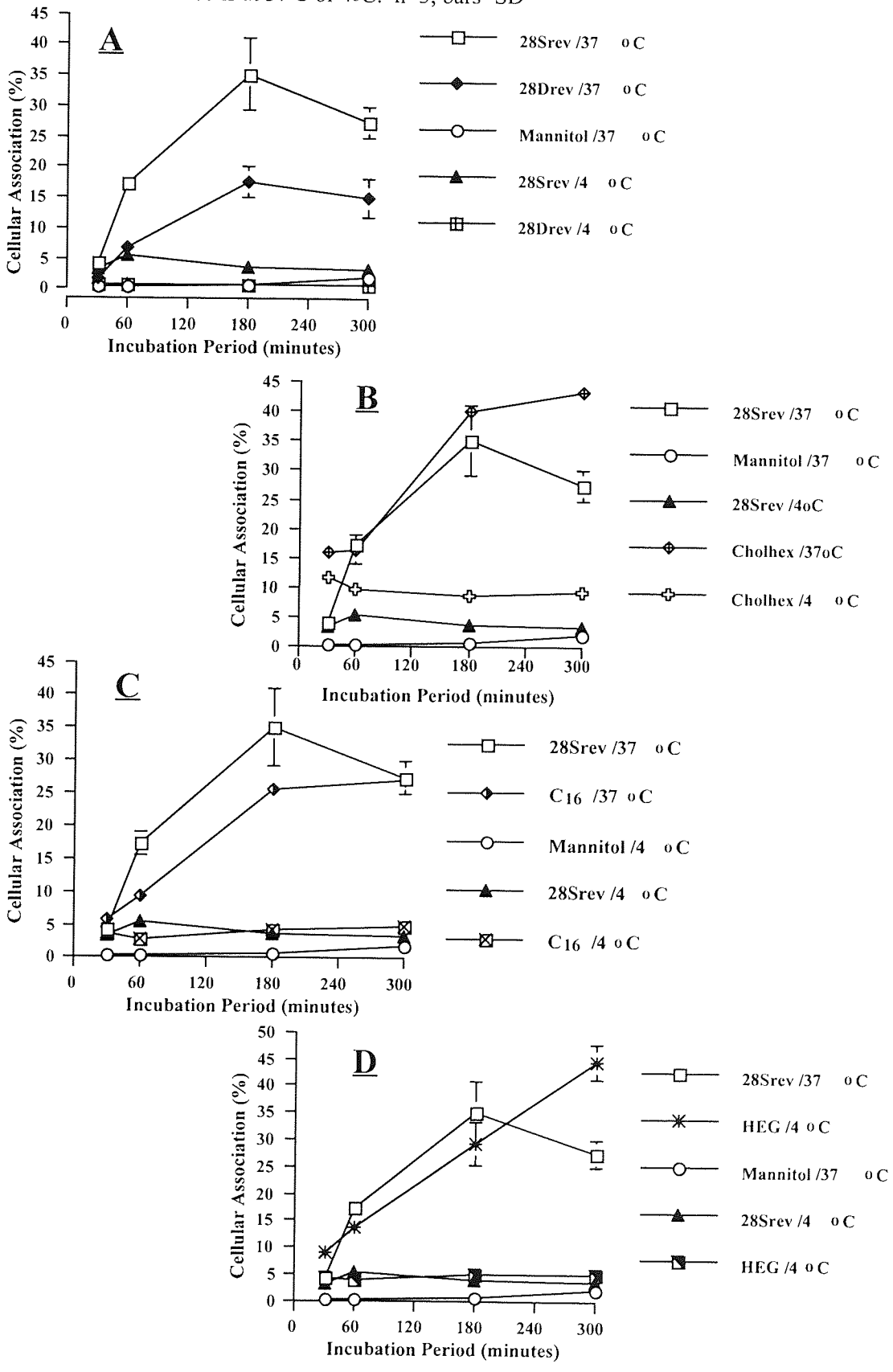
Both 28Drev and 28Srev ODNs bound RAW264.7 cells to a significantly higher degree at 37°C than 4°C, $p<0.0001$ and $p=0.007$ for 30 minutes incubation and $p=0.002$ and $p<0.0001$ for 300 minutes incubation respectively. Both ODNs also bound RAW264.7 cells to a significantly higher degree than mannitol at 37°C, $p<0.0001$ and $p<0.0001$ for 30 minutes incubation and $p=0.030$ and $p<0.0001$ for 300 minutes incubation, for 28Drev and 28Srev respectively. The profiles for mannitol at 37°C, 28Srev and 28Drev ODNs at 4°C, were similar. This data suggests that different mechanisms were involved in cellular association of the ODNs and mannitol when both are incubated 37°C.

The cellular association profiles at 37°C and 4°C for the cholhex conjugate (figure 4.11B) also showed significant temperature dependency, 30 ($p=0.0004$) and 300 ($p<0.0001$) minutes incubation at the respective temperatures. There was a significantly higher degree of cholhex cellular association compared to mannitol, 30 ($p<0.0001$) and 300 ($p<0.0001$) minutes incubation respectively. As with the unconjugated ODNs, the profile at 4°C showed a similar pattern to that of mannitol, a low constant level of association occurring at all the incubation periods, *i.e.* no accumulation. Cholhex had a higher cellular association compared to the control 28Srev at all the incubation periods and the basal level of binding was higher than 28Srev at 4°C.

As shown in figure 4.11C, C_{16} conjugate cellular association was significantly temperature dependent, for incubations at 37°C and 4°C after 30 ($p=0.001$) and 300 ($p<0.0001$) minutes respectively. We can therefore conclude that cellular energy was required for efficient conjugate binding. The cellular association profiles for the C_{16} conjugate and mannitol were significantly different, ($p<0.0001$) for 30 and 300 minutes incubation respectively.

As shown in figure 4.11C, C_{16} conjugate cellular association was significantly temperature dependent, for incubations at 37°C and 4°C after 30 ($p=0.001$) and 300 ($p<0.0001$) minutes respectively. We can therefore conclude that cellular energy was required for efficient conjugate binding. The cellular association profiles for the C_{16} conjugate and mannitol were significantly different, ($p<0.0001$) for 30 and 300 minutes incubation respectively.

Figure 4.11 (A, B, C and D) : Cellular Association of 28Srev, Conjugate ODNs and Mannitol with RAW264.7 Cells. Graph to show the cellular association (%) of the 28Srev ODN, 3H-mannitol and (A) 28Drev, (B) Cholhex, (C) C_{16} and (D) HEG, with RAW264.7 cells at 37oC or 4oC. n=3, bars=SD



The C_{16} conjugate did not associate with the cell to a higher degree than the control 28Srev, although at 300 minutes cellular association values were very similar (figure 4.11C). Stability of the C_{16} conjugate at 300 minutes incubation with the RAW264.7 cells was found to deteriorate (section 4.3.2.1, figure 4.4). Therefore this cellular association profile, after 180 minutes incubation, may not be a true representation of intact C_{16} conjugate due to degradation.

HEG cellular association was, like the other Srev ODNs, highly temperature dependent and markedly different from mannitol (figure 4.11D). The statistical difference in cellular association for HEG at 37°C and 4°C was significant, $p<0.0001$ for both 30 minutes and 300 minutes. Comparing HEG cellular association with mannitol, again there was a significant difference, $p<0.0001$ after 30 minutes and 300 minutes incubation at 37°C. The HEG conjugate bound RAW264.7 cells to a greater degree than the control 28Srev after 300 minutes incubation at 37°C (figure 4.12D), cellular association at the shorter incubation periods was lower than the 28Srev. However, as shown in section 4.3.2.1, the HEG conjugate showed degradation at 300 minutes incubation with RAW264.7 cells, therefore the values for cellular association at this time period may have represented ODN fragments, free ^{32}P-ATP and intact HEG conjugate.

The length of the ODN may have masked the effect of the lipophilic HEG group accounting for the lower degree of cellular association of the HEG conjugate compared to 28Srev ODN. An attempt was made to shorten the HEG conjugate, a 14mer-HEG conjugate produced and cellular association compared to a 14Srev ODN. Both ODNs were found to have similar cellular associations (data not shown), therefore length of the ODN was not a factor in the cellular association and the HEG moiety did not enhance binding.

All the Srev ODNs showed distinct temperature dependency, incubation at 4°C resulted in a marked decrease in cellular association, a constant low level of association was seen between 30 minutes and 300 minutes incubation. This data therefore indicates that the mechanisms involved in cellular association at the two

temperatures were distinctly different, physiological temperature necessary for efficient association. It can be inferred from the data that mannitol, at 37°C, and the Srev ODNs, at 4°C, associate with the macrophages by similar mechanisms.

Cellular association of the ODNs at 37°C increased with time, the cholhex, C_{16} and HEG conjugates showed a continuing increase in whereas the control 28Srev ODN appeared to plateau, however, the standard deviations indicate that this plateauing may have started at the 180 minute period.

4.3.2.6 Efflux of Srev Oligonucleotides From Cells

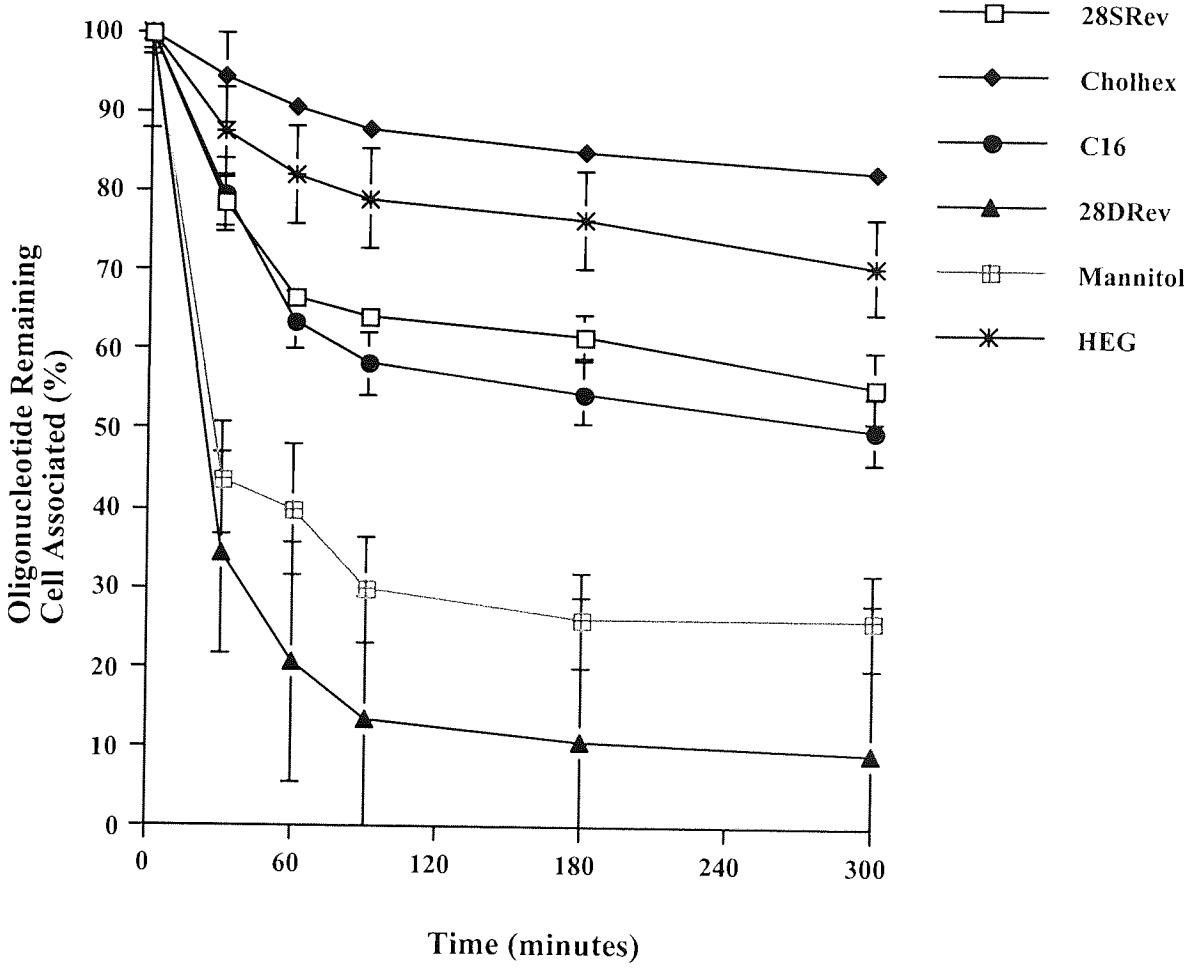
Efflux of molecules from cells following internalisation is discussed in section 2.5.2.1, those molecules internalised passively generally having the fastest efflux rate. Studies have shown fluid phase markers (Besterman *et al.* 1981) and ODNs (Wu Pong *et al.* 1992; Temsamani *et al.* 1994; Tonkinson *et al.* 1994) to efflux from cells, at differing rates depending on their method of internalisation.

To obtain a clearer profile of the association characteristics of each of the Srev ODNs with RAW264.7 and A431 cells, efflux from cells into the culture medium was assessed over a 4 hour time period at 37°C (protocol described in section 2.5.2.1) and compared with that of the fluid phase marker mannitol. A slower rate of efflux for the conjugates in comparison to unconjugated 28Srev may be indicative of a higher predisposition to remain internalised. ODNs, which are retained within cells for a longer period of time, may have an improved likelihood of reaching their target and exerting an antisense effect. Efflux of the various *rev* ODNs and mannitol is shown in figure 4.12 from RAW264.7 cells, and figures 4.13 for A431 cells.

Both cholhex and HEG conjugates had a slower efflux rate than to the 28Srev ODN, 82.6% of cholhex and 70.7% of HEG remained associated with RAW264.7 cells after 4 hours incubation (figure 4.12). The cholhex was either internalised into deep compartments (Tonkinson *et al.* 1994) or very tightly bound to the cell, only 17.3%

lost over 4 hours at 37°C. After the same time period only 55.6% of the 28Srev and 50.2% of C_{16} remains cell associated. Therefore the C_{16} conjugate was most rapidly effluxed from the cell.

Figure 4.12: Efflux of the 28Srev and Conjugate ODNs and Mannitol from RAW264.7 Cells. Graph to show efflux 28Drev, 28Srev, conjugated ODNs and mannitol from RAW264.7 cells over time. Data is represented as the % ODN remaining associated with cells at various times, following an initial 1hr incubation period at 37°C. n=4, bars=SD.



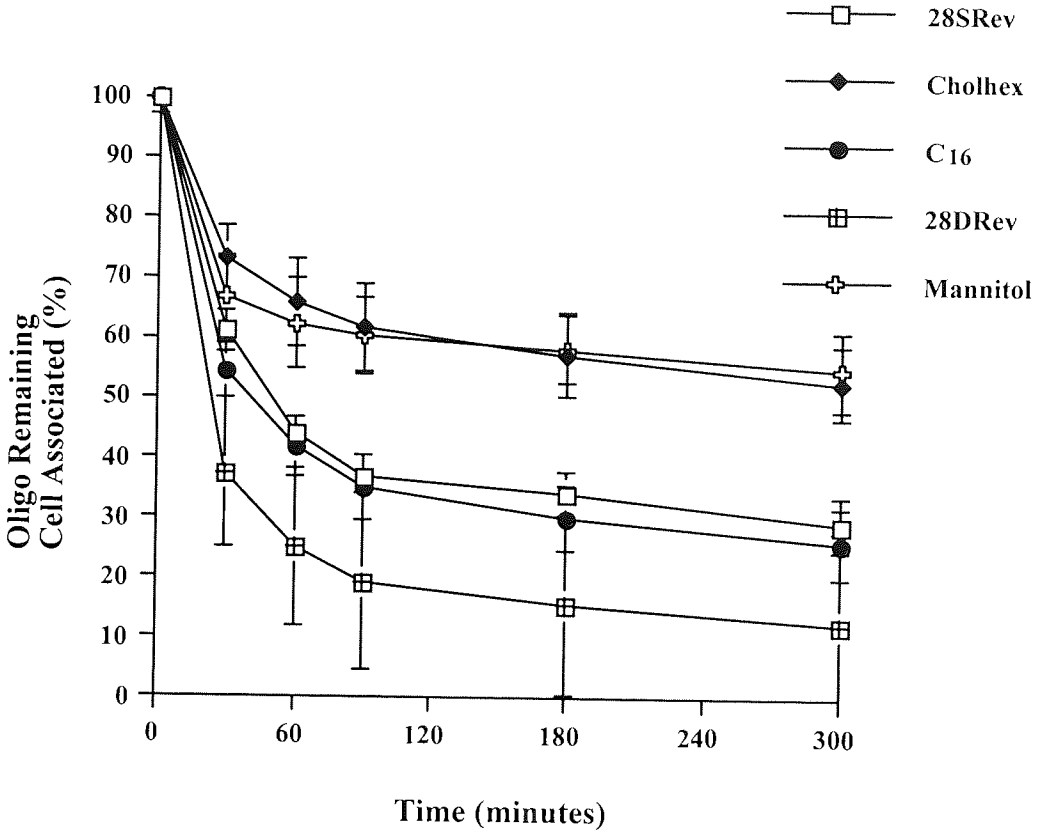
Efflux of the fluid phase marker mannitol, from RAW264.7 cells (figure 4.12), was faster than for the conjugates and 28Srev ODNs, more than 50% was effluxed after the first 30 minutes, only 26.0% remaining after 5 hours, compared to over 50% remaining for all the ODNs. The 28Drev was also effluxed quickly from the cells, the

profile being similar to mannitol, 34.4% remaining after 60 minutes and only 9.5% after 300 minutes. This data is consistent with Tonkinson *et al.* (1994) who report the efflux of PO-ODNs to be faster than that of PS-ODNs, concluding this to be a result of internalisation into shallow and deep compartments respectively.

Efflux of the Srev ODNs out of A431 cells was faster compared to the RAW264.7 macrophages. The cholhex conjugate had the slowest rate of efflux from RAW264.7 cells, only 26.9% was lost after 60 minutes (figure 4.13), compared to 17.3% in A431 cells. As with the RAW264.7 cells, the efflux profiles of the 28Srev and C_{16} ODNs were similar, 36.9% and 35.0% remaining associated respectively after 300 minutes.

The efflux profile for mannitol (figure 4.13) from A431 cells was very different from that in RAW264.7 cells, 54.9% remained associated after 300 minutes whereas only 26.0% remained in RAW264.7 cells, a slower efflux than even the 28Srev, 28Drev ODNs and C_{16} conjugate. This was an unexpected observation as fluid phase markers are characteristically effluxed quickly from cells due to the establishment of equilibrium between endocytosis and exocytosis (Besterman *et al.* 1981).

Figure 4.13: Efflux of the 28SRev, 28DRev, Conjugate ODNs and Mannitol from A431 cells. Graph to show efflux of 28SRev, 28DRev, Conjugate ODNs and Mannitol from A431 cells over time. Data represented as the % ODN remaining cell associated at various times after an initial 1hr incubation period at 37oC. n=4, bars=SD

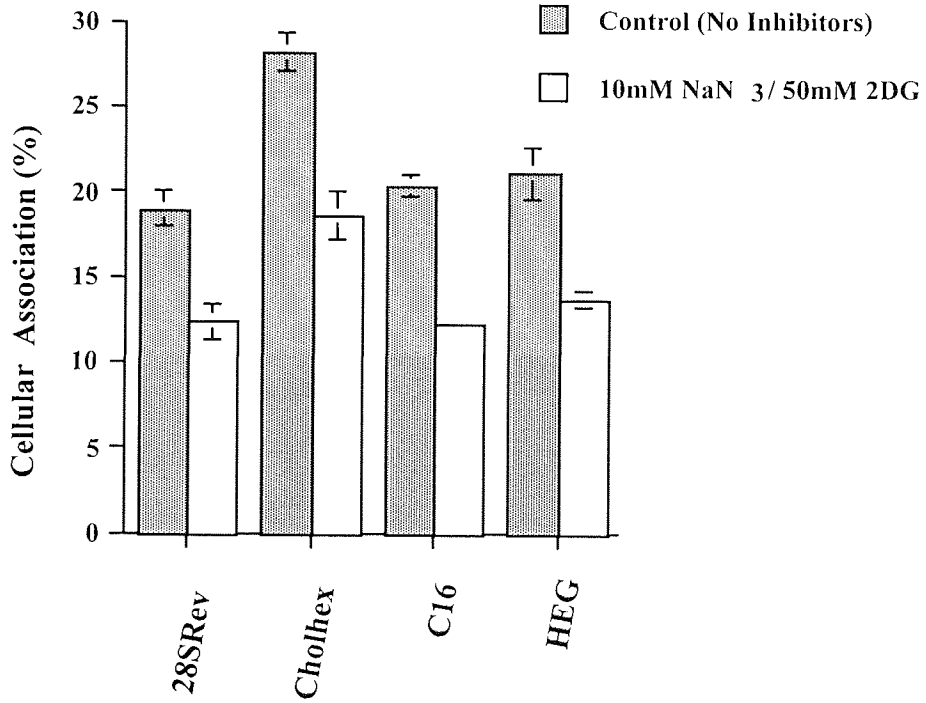


4.3.2.7 The Effect of Metabolic Inhibitors

Section 4.3.2.5 describes the effect of temperature on the cellular association of all the Srev ODNs. Cells incubated at 4°C bound significantly less ODN than those incubated at physiological temperature, implying an active, energy requiring mechanism. Metabolic inhibitors were used to further confirm the energy dependent nature of these processes (Loke *et al.* 1989; Wu Pong *et al.* 1992 and 1994a; Gao *et al.* 1992; Beltinger *et al.* 1994). Sodium azide (NaN_3) is an inhibitor of the enzyme cytochrome oxidase, an essential respiratory enzyme complex found in the inner mitochondrial membrane (section 2.5.2.4). 2-deoxy-glucose (2DG) is an inhibitor of

glycolytic cycle (Alberts *et al.* 1986). RAW264.7 cells were pre-incubated with NaN_3 and 2DG as described in section 2.5.2.4, prior to ODN addition. Cellular association was then assessed following the standard protocol, compared to a control in the absence of inhibitors (figure 4.14).

Figure 4.14: Effect of Metabolic Inhibitors on Cellular Association of the 28Srev Oligonucleotide and Conjugates. Graph to show the cellular association (%) of the Srev ODNs in the presence and absence of the metabolic inhibitors, sodium azide (NaN_3, 10mM) and 2-deoxy-glucose (2DG, 50mM)following incubation for 1hr at 37°C with RAW264.7 cells. n=4, bars=SD.

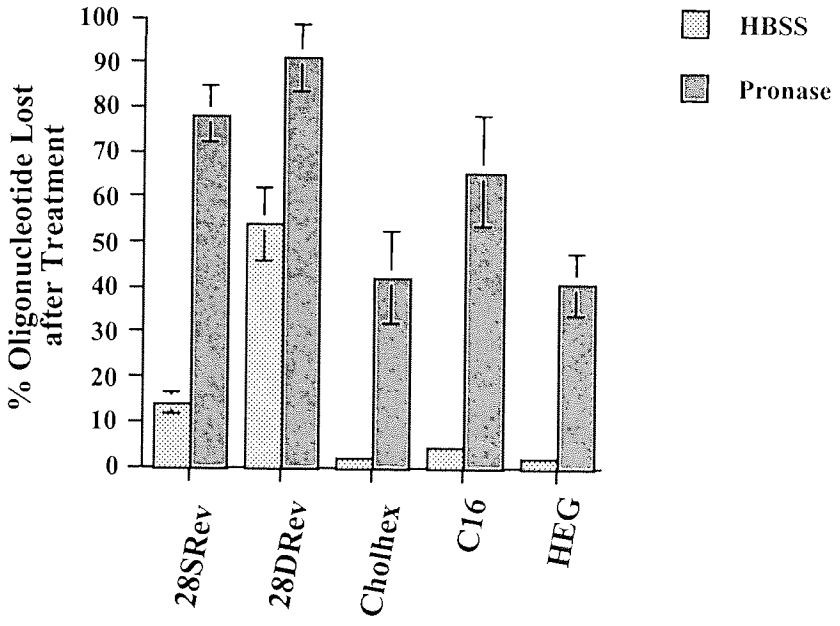


Shown in figure 4.14 is the cellular association data obtained after a 1 hour incubation of ODNs with RAW264.7 cells, in the presence and absence of inhibitors. The cellular association of all the ODNs was significantly ($p<0.0001$) reduced in the presence of NaN_3 and 2DG, all to similar degrees. As demonstrated for the effects of temperature in figures 4.1, ODN association was not completely abolished under conditions, which inhibit active uptake processes, this is in agreement with Gao *et al.* (1992).

4.3.2.8 The Effect of the Proteolytic Enzyme Pronase

Pronase is able to degrade cell surface proteins, thereby any protein associated molecules, such as ODNs, are also removed. Any depletion in cellular association after Pronase treatment can be attributed to the degradation of proteins binding ODNs. Cells were co-incubated with each ODN for 60 minutes at 37°C (section 2.5.2.6) prior to a 15 minute incubation of the cells with 0.25% Pronase at 4°C. As a control, cells were also incubated with HBSS for 15 minutes in parallel to those in Pronase. The amount of ODN lost in either the Pronase or control HBSS wash was calculated, shown in figure 4.15.

Figure 4.15 : Percentage of 28Srev Oligonucleotide and Conjugates Lost Following HBSS or Pronase Treatment. Graph to show the amount (%) of Srev ODN stripped off RAW264.7 cells by a 15 minute treatment with either HBS or 0.25% pronase (at 4C) following an initial incubation period of 1hr at 37. n=4, bars=SD



The unconjugated 28Srev ODN was most affected, 78.5% of this ODN was removed by the Pronase treatment compared to 14.0% by a HBSS wash ($p<0.0001$). Pronase treatment stripped off 65.8% of the C_{16} conjugate ($p<0.0001$), significantly more than the other conjugates. 42.2% of cholhex was stripped off ($p<0.0001$) and 40.9% of

HEG ($p<0.0001$), significant differences in the amount of ODN removed between Pronase and HBSS treatment. The phosphodiester, 28Drev, was also affected by Pronase, 90.8%% stripped off in by Pronase treatment compared to 54.1% by HBSS, not as statistically significant as the PS-ODNs ($p=0.002$).

It appears that the 28Srev ODN was bound to surface proteins to the greatest extent. At the shorter incubation periods, under 180 minutes, the 28Srev ODN has a higher cellular association compared to the C_{16} and HEG conjugates (figures 4.11A, 4.11C and 4.11D respectively), the conjugate moieties not appearing to enhance 28Srev ODN binding to the cells. However this Pronase wash data shows that a considerable amount of the 28Srev ODN is only surface bound to proteins on the cell, therefore although there may be a greater quantity binding compared to the other conjugates, this quantity is not necessarily internalised.

Pronase less affected the conjugate ODNs. This could be attributed to their binding proteins which are less Pronase-sensitive, or non-protein surface molecules such as carbohydrates or lipids, or that the ODNs are internalised within the cell. The conjugate most affected by Pronase was C_{16}, approximately 4-fold more was stripped off compared to the cholhex and HEG conjugates. This indicates that cholhex and HEG are binding to the RAW264.7 cells more efficiently than the C_{16}, possibly internalised.

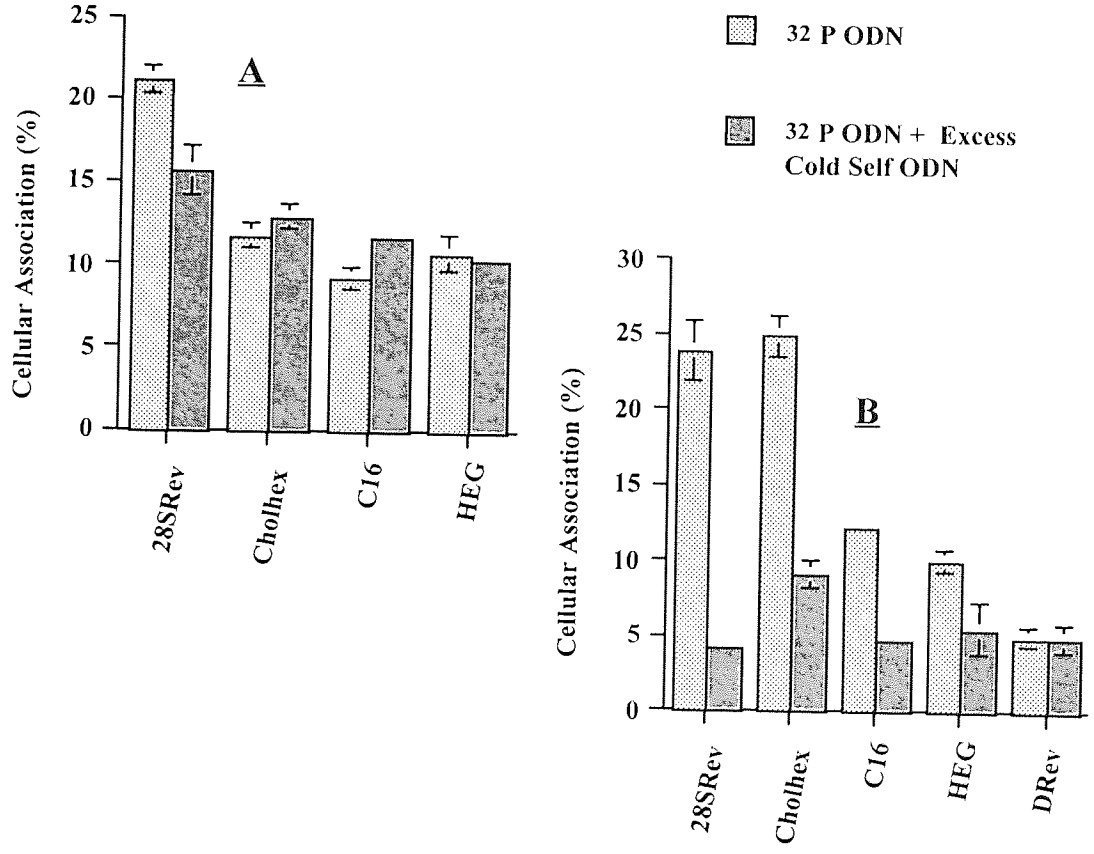
Suprisingly the cellular association of 28Drev was also significantly reduced by Pronase treatment, as phosphodiesters do not generally bind proteins to the same extent as phosphorothioates. An explanation for such a reduction could be that the 28Drev binds non-specifically to surface proteins via ionic interactions, in the same way as PS-ODNs, therefore Pronase sensitive surface protein removal reduces the fraction of 28Drev associated.

4.3.2.9 The **Effect of Self Competition**

Each of the Srev ODNs was assessed for binding saturation on the surface of RAW264.7 cells by the addition of a 100-fold excess of cold ODN (section 2.5.2).

Figure 4.16A shows the effect of self-competition on cellular association of the Srev ODNs. In the presence of 100-fold excess cold self-ODN only the cellular association of the 28Srev and C_{16} were significantly reduced ($p=0.0002$ and $p=0.0004$ respectively), the cholhex and HEG conjugate ODNs were not affected.

Figure 4.16 (A and B) : Effect of Self Competition of on Cellular Association of the 28Srev Oligonucleotide and Conjugates. Graph to show the effect on cellular association (%) of the Srev ODNs in the presence and absence of cold self-ODN following 1hrs incubation at 37°C with RAW264.7 Cells. 100-Fold excess self-ODN (4.17A) and 250-fold excess self-ODN (4.17B). n=4, bars=SD



Cold ODN at a concentration of 10μM did not affect cellular association of the ^{32}P-labelled fraction for the conjugates. This observation can be interpreted in two ways, either saturation of surface receptors was not reached by 100-fold excess or no specific receptors exist on the RAW264.7 cell surface. To clarify this, a higher concentration of cold self-ODN (25μM) was used.

A significant reduction in cellular association, for all the *rev* ODNs, was seen when the labelled fraction was competed with a 250-fold excess of cold ODN (figure 4.16B). The PO-ODN, 28Drev, was not affected by the presence of a 250-fold excess of self ($p=0.409$). Cellular association of 28Srev was reduced by 82% ($p<0.0001$), a 3-fold higher depletion than in the presence of 100-fold excess unlabeled self. Cellular association of all the Srev conjugates was significantly affected by self-competition, a 63% reduction in cholhex ($p<0.0001$), 60% reduction of C_{16} ($p<0.0001$), and 44% reduction of HEG cellular association ($p<0.0001$). At this higher concentration the RAW264.7 cells saw saturation, indicating a limited binding capacity for all the ODNs.

Cellular association of the 28Srev was most significantly reduced in the presence of self-competition indicating that the binding capacity of RAW264.7 cells is lower for this ODN than the conjugates. Saturation is reached at a lower concentration, implying fewer binding sites. Cholhex showed a greater reduction in cellular association than the other conjugates, indicating that RAW264.7 cells have relatively fewer binding sites for this conjugate. HEG was least affected by self-competition.

4.3.2.10 The Effect of Competition with 28Srev Oligonucleotide

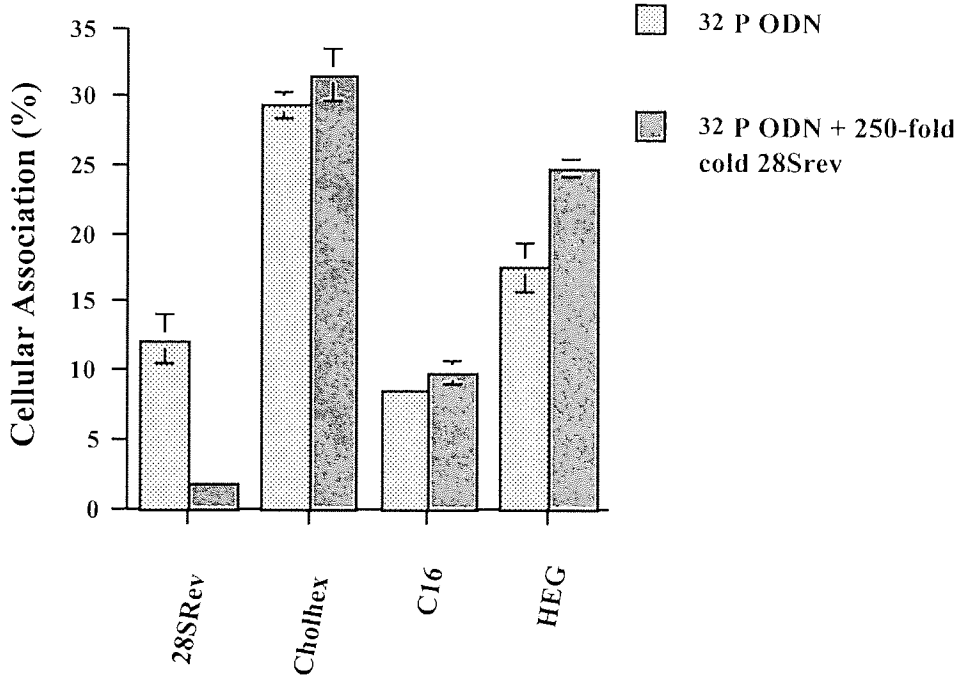
Self-competition data established that there is a limited binding capacity for each of the conjugates on RAW264.7 cells. Competition with excess cold 28Srev ODN was used to address the issue of whether the conjugate or the ODN moiety was involved in cellular association. If 28Srev ODN can compete for cellular association of a

conjugate then it is likely that they share a binding site on the cell, conferred by the Srev ODN portion. If competition is not seen then binding may is attributed to the conjugate moiety. A 250-fold excess of cold 28Srev ODN was used (figure 4.17).

As shown in figure 4.17 there was no significant decrease in cellular association for the cholhex and C_{16} conjugates, 7.3% ($p=0.21$) and 15.6% ($p=0.011$) increases respectively. However, cellular association of the 28Srev was decreased significantly, by 85.0% ($p<0.0001$). Cellular association of HEG conjugate in the presence of the 250-fold 28Srev increased by 41.9% ($p<0.0001$).

From this data we can deduce that a 250-fold excess of 28Srev did not inhibit the cellular association of the conjugates indicating that the conjugates bind to different sites than the 28Srev ODN on RAW264.7 cells.

Figure 4.17 : Effect of Competition with the 28Srev Oligonucleotide on Cellular Association of 28Srev Oligonucleotide and Conjugates. Graph to show the effect on cellular association (%) of the Srev ODNs of the presence and absence of a 250-fold excess cold 28Srev ODN following 1hrs incubation at 37C with RAW264.7 cells. n=4, bars=SD

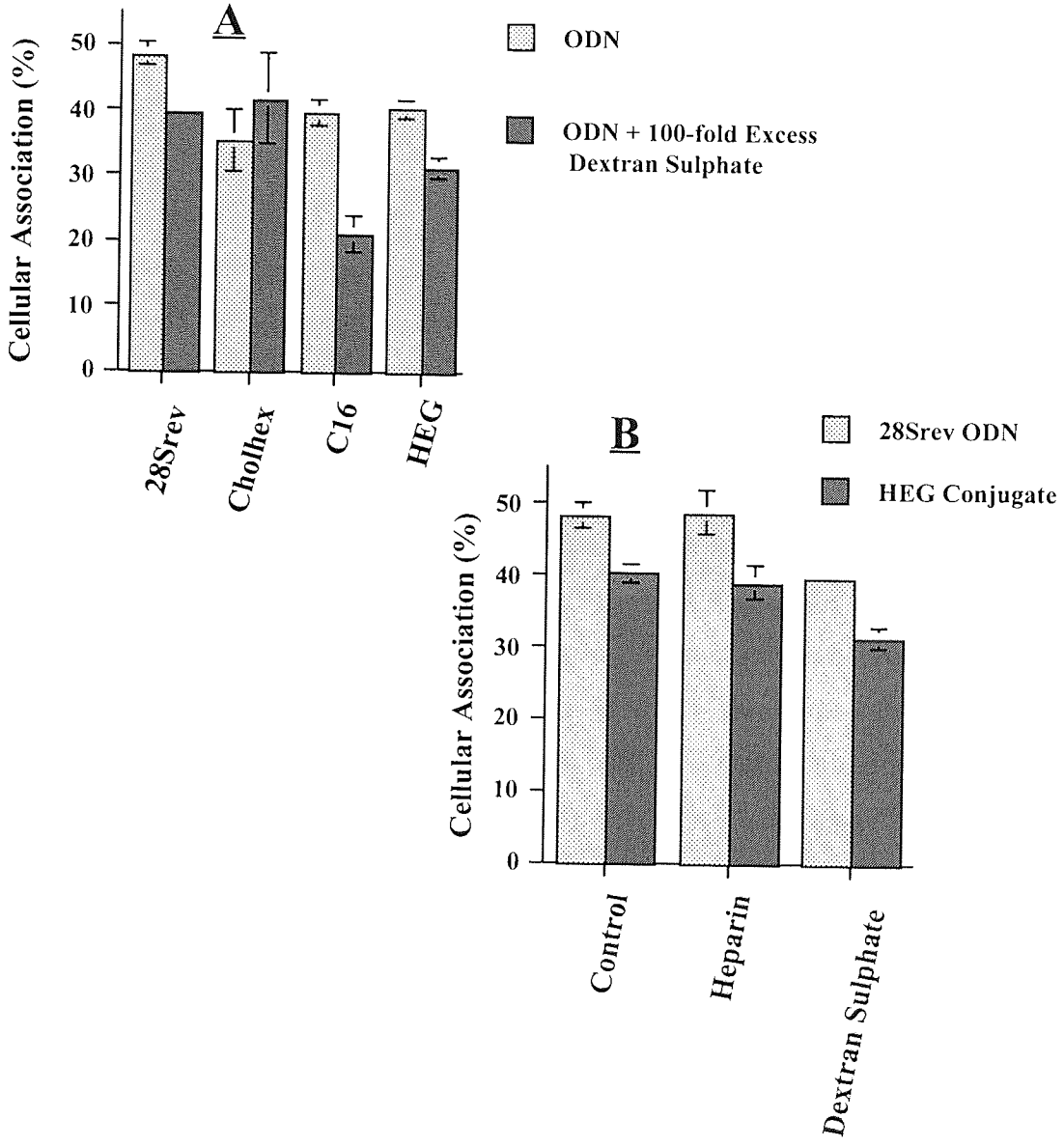


4.3.2.11 The Effect of Competition with Polyanions

Cross competition of the Srev conjugates was not seen in the presence of excess 28Srev ODN which indicated that the lipophilic moiety has a central role in binding to the RAW264.7 cells. The effect of cross competition with polyanionic, non-nucleic acid molecules was assessed (section 2.5.2.8) in order to ascertain the nature of the interaction between the conjugate and the cell. Cells were incubated with each Srev ODN for 4 hours at 37°C following a 15 minute pre-incubation period between the cells and 100-fold excess dextran sulphate. The results are shown in figure 4.18A.

The cellular association of cholhex was not affected by the presence of a 100-fold excess of dextran sulphate (figure 4.18A). Interaction of the cholhex conjugate with cells appeared to be more specific, either occurring via a strong ionic interaction or an independent mechanism. Cellular association of the C_{16} conjugate was inhibited most significantly ($p<0.0001$), whilst cellular association of 28Srev and HEG were depleted to a lesser extent, 19% ($p<0.0001$), and 22% ($p<0.0001$) respectively. Inhibition by dextran sulphate indicated that the association of the latter three ODNs was of an ionic nature and therefore not very specific.

Figures 4.18 (A and B) : Effect of Competition with Polyanions on the Cellular Association of the 28Srev Oligonucleotide and Conjugates . Graph to show the cellular association (%) of the Srev ODN and conjugates in the presence and absence of a 100-fold excess of the polyanions, following 4 hours incubation at 37ºC. 100-Fold excess of Dextran Sulphate (4.19A), 100-fold excess of dextran sulphate or heparin (4.19B). n=4, bars=SD



Two ODNs were selected for further evaluation. The effects of competition between the control 28Srev and HEG conjugate with other polyanions were also evaluated. Heparin was used as a competitor at 100-fold excess (section 2.5.2.8), and compared to the data for dextran sulphate. Unlike dextran sulphate, heparin had no significant

affect on cellular association at this concentration (100-fold excess, 10μM), shown in figure 4.18B. Heparin, has approximately a 50% higher MW than the two ODNs. Theoretically heparin will also compete for ionic interactions with cell surface proteins, as shown for dextran sulphate.

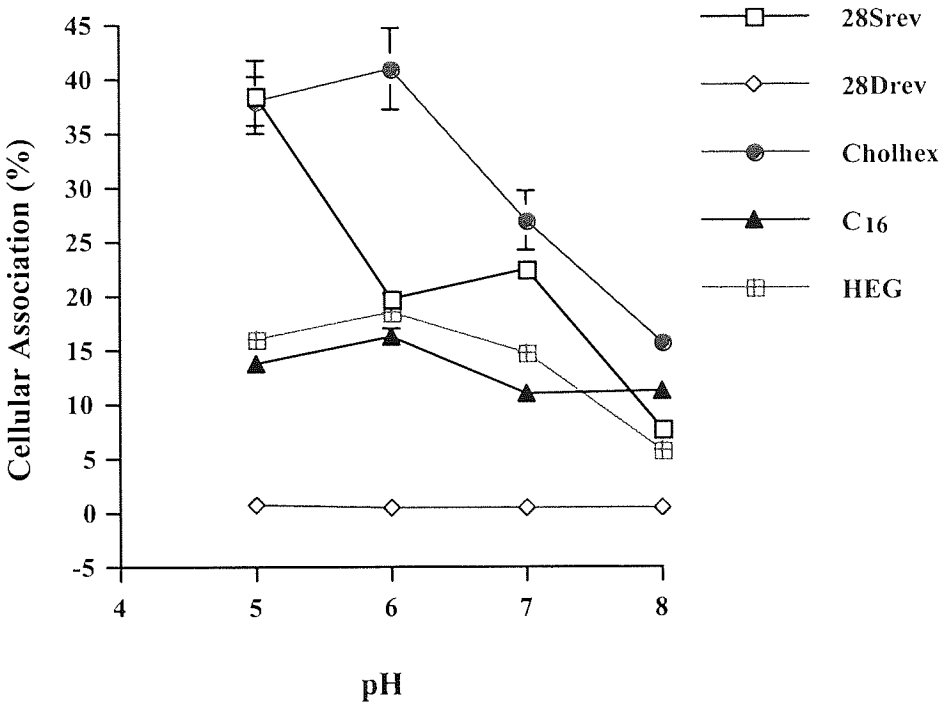
4.3.2.12 The Effect of pH

The exogenous pH of cells is a very important factor when considering association of ligands with cell surface membrane proteins (section 2.5.2.5). Proteins become protonated as pH is decreased, making them more cationic and therefore more attractive to anionic molecules such as ODNs.

If the Srev ODNs are associating with cells via interactions with proteins, then a change in pH should affect the degree of cellular association (Hawley *et al.* 1996). The pH of the cell culture media (HBSS or MES) was adjusted and cells incubated with the Srev ODNs for 1 hour at 37°C according to the standard protocol. All previous experiments were performed at pH7.2. Figure 4.19 shows the associations of ODNs with RAW264.7 cells at pH 5.0, 6.0, 7.0 and 8.0. Comparisons were made between each of the lipophilic conjugates and the control Srev ODN, the 28Drev ODN also evaluated.

The cholhex conjugate had the highest association (with RAW264.7 cells) over the range of pH studied. Values were similar at pH 5.0 and 6.0, but significantly decreased as the pH further increased to 7.0 (by 34.1% from pH6.0 to 7.0) and further increased by 41.6% from pH7.0 to 8.0.

Figure 4.19 : Effect of pH on Cellular Association of the 28Srev Oligonucleotide and Conjugates. Graph to show the cellular association (%) of the Srev ODNs with RAW264.7 cells incubated at different pH for 1 hour at 37oC. n=3, bars=SD



Association of the 28Srev ODN differed from the cholhex conjugate in that a significant decrease occurred from pH5.0 to 6.0 as opposed to occurring at pH7.0. Therefore, the proteins with which the 28Srev ODN associates may be more pH sensitive, more readily de-protonated as the pH increased from 5.0 to 6.0, and as the pH was further decreased from 7.0 to 8.0, cellular association decreased very significantly by 66.0%. Overall, from pH5.0 to 8.0, the 28Srev cellular association was decreased significantly by 80.2% ($p<0.0001$), compared to a 58.4% decrease for the cholhex conjugate ($p=<0.0001$).

The C_{16}, HEG and 28Srev ODNs showed very little change in cellular association over the pH range 5.0 to 8.0, compared to the other ODNs (figure 4.19). From pH5.0 to 8.0 there was an overall decrease in C_{16} association by 28.8% ($p=0.020$), and therefore the effect of pH on cellular association was less significant than for the 28Srev and

cholhex ODNs. It can be inferred from this data that the C_{16} conjugate either binds proteins to a lesser extent or binds relatively pH insensitive proteins.

The HEG conjugate showed the expected pattern of cell association, decreasing as the pH increased. The overall change was 64.8% from pH 5.0 to 8.0 ($p=0.026$), again an less significant change than for the 28Srev and cholhex ODNs. Compared to C_{16}, the HEG appears protein bound to a greater extent or the proteins involved are more pH sensitive.

28Drev cellular association was also found to be affected by pH change, in the same way as the phosphorothioate counterpart. Phosphodiesters have previously been reported to be protein bound by a number of studies (Goodarzi *et al.* 1991; Nakai *et al.* 1996), their cellular association decreasing with increasing pH. An overall decrease in cellular association, of 48.6%, was observed from pH5.0 to 8.0 ($p=0.001$).

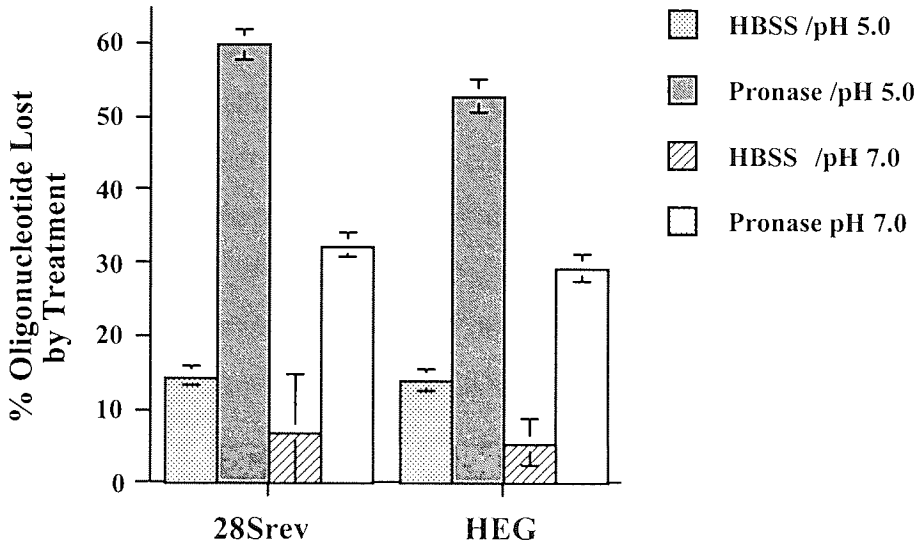
This data implies that the various ODNs studied were associating with the RAW264.7 cells via protein interactions, association increasing as the proteins become protonated at lower pH, but to differing degrees. If the decrease in cellular association from pH5.0 to 8.0 is taken as an indicator of protein binding, the 28Srev ODN was most affected (by 80.2%), followed by HEG (by 64.8%), cholhex (58.4%) and the C_{16} conjugate (by 26.3%). It appears that both PO and PS backbones are affected by pH, however cellular association of the PS-ODN was more pH sensitive, as this backbone binds proteins to a greater extent.

To further evaluate the degree of 28Srev and HEG ODN binding to RAW264.7 membrane surface proteins, the effect of Pronase washing was evaluated on cells incubated at pH5.0, compared to those incubated at pH7.0 (figure 4.20). As described in section 2.5.2.5, cells were incubated with the ODN at pH 5.0 and 7.0, and subsequently treated with Pronase as described in section 2.5.2.6.

Figure 4.20 shows that a greater amount of ODN, either 28Srev and HEG, was stripped off by Pronase at pH5.0, compared to pH7.0, and compared to the HBSS

wash. At pH5.0, the cellular association of 28Srev and HEG increased, Pronase treatment subsequently removing more ODN.

Figure 4.20: Percentage 28Srev Oligonucleotide and HEG Conjugate Lost following HBSS or Pronase Treatment. Graph to show the amount (%) of 28Srev or HEG ODN stripped off RAW264.7 cells by a 15 minute treatment with either HBSS or 0.25% Pronase/HBSS, incubation at the stated pH for 1 hour at 37ºC. n=4, bars=SD



We can conclude that a decrease in pH does cause increased association to proteins, which is reflected by the data from Pronase washing, the increase in amount of ODN stripped at pH5.0 compared to pH7.0 (figure 4.20). These findings fit with the theory that a cell surface binding molecule is involved in binding of the 28Srev and HEG ODNs.

4.3.2.13 The Effect of Endocytosis Disruption

The fluorescent micrographs obtained from incubation of the RAW264.7 cells with a cholesterol conjugated ODN (section 4.3.2.2, figures 4.6A, B, C and D) revealed a punctate distribution of the conjugate, indicating internalisation into intracellular vesicles, possibly endosomes or lysosomes, with very little localisation to the nucleus. A receptor-mediated mechanism was also demonstrated by temperature dependence, sensitivity to the proteolytic enzyme Pronase, cellular association inhibition by metabolic inhibitors and a higher degree of cellular association compared to a fluid phase marker. Monensin (section 2.5.2.9), an agent which disrupts the endocytosis cycle, was used to further investigate the cellular association and uptake mechanism of the Srev ODN and conjugates. Monensin may have a number of effects on the cellular association of ODNs if the uptake pathway following binding involves acidic compartments. The reagent may cause a decrease in the cell surface receptor number, or cellular association may increase as ODN is internalised and trapped in the acidic compartments.

Cells were treated with monensin as described in section 2.5.2.9, being pre-incubated with monensin for 30 minutes prior to addition of the ODN and then further incubated for 4 hours.

Shown in figure 4.21A is the cellular association data obtained in the presence and absence of monensin. All the Srev ODNs were significantly affected by the monensin, cellular association of all ODNs decreased by approximately 50% ($P<0.0001$).

This data leads to the conclusion that monensin disrupts the association of each of the ODNs with RAW264.7 cells. Monensin could either be having an effect on the Srev ODNs within the cell in terms of intracellular trafficking or maybe on the surface of the cell before internalisation occurs. To investigate whether monensin was having an effect at the surface of the cell or internally, two further experiments were performed using the 28Srev ODN and HEG conjugate. Cells were co-incubated with monensin

and the ODN for a shorter period (30 minutes at 37°C), the pre-incubation period omitted and the standard cell association protocol followed (section 2.5.2).

Figures 4.21 (A and B) : Effect of Monensin on the Cellular Association of the 28Srev Oligonucleotide and Conjugates. Graph to show the cellular association (%) of the Srev ODN and conjugates in the presence and absence of 10M monensin with RAW264.7 cells, incubated at 37C. Cells either (A) pre-incubated with monensin for 30 minutes prior to ODN addition and a further 4 hrs incubation or (B) cells co-incubated with monensin and ODN for 30 minutes (B). n=4, bars=SD.

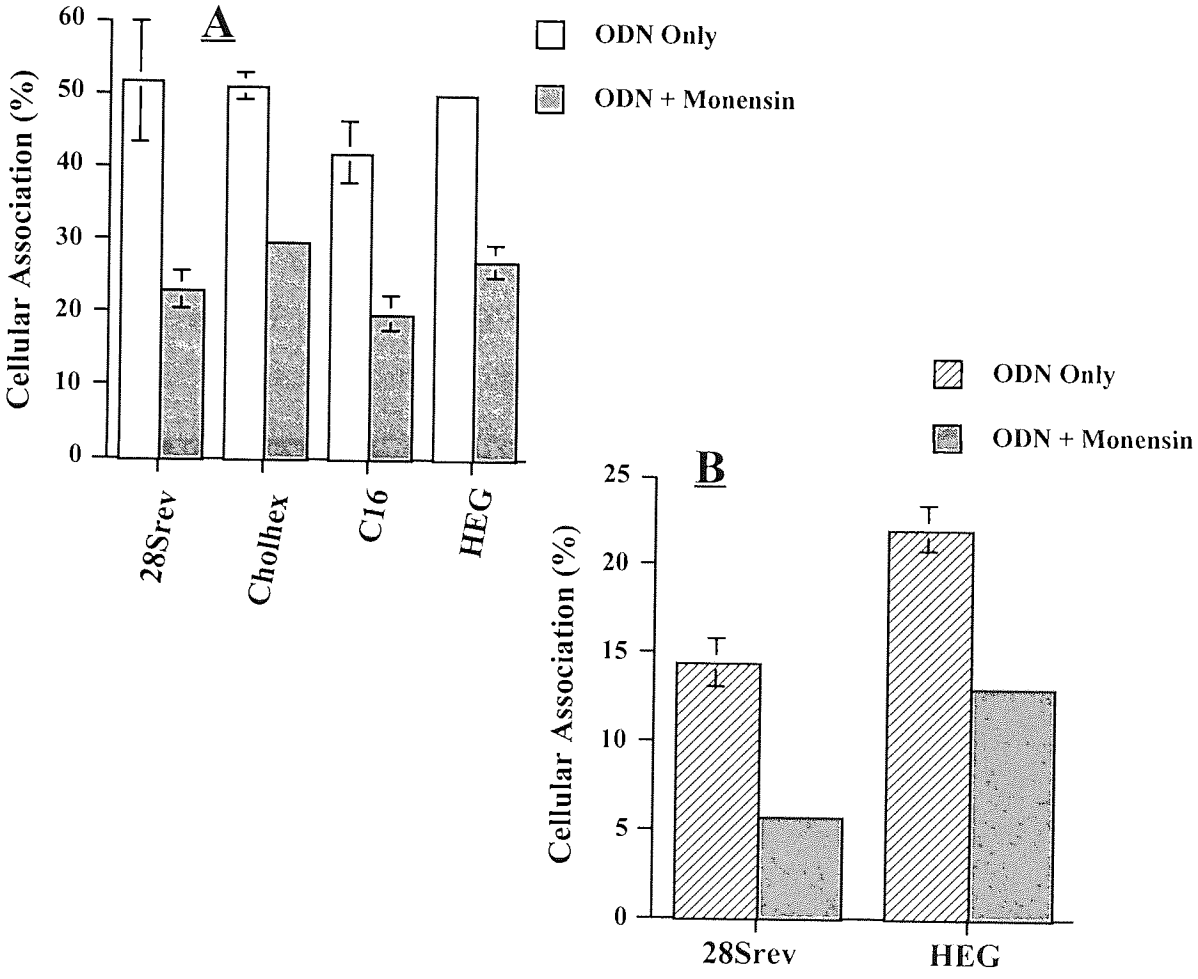


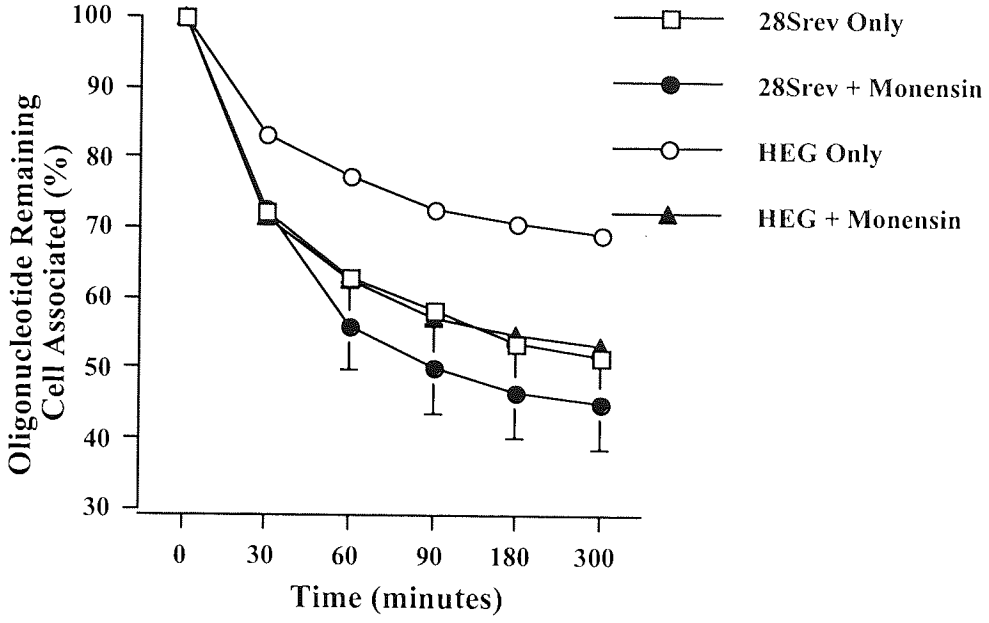
Figure 4.21B shows the effect on cellular association of co-incubation of monensin with 28Srev or HEG after 30 minutes incubation. Co-incubation of the ODNs with monensin in RAW264.7 cells resulted in a decrease in cellular association of both the 28Srev and HEG ODNs, by a similar extent to that demonstrated in the previous

experiment. Therefore, it appears that monensin is having an effect at the surface of the cell, or that any membrane recycling that occurs will be at a fast rate.

The effect of monensin on efflux of the 28Srev and HEG ODNs from RAW264.7 cells was also investigated, to determine whether intracellular trafficking inside the cell was disrupted by monensin. Acceleration in the efflux rate of ODNs would appear as a decrease in cellular association. Cells were pre-incubated with monensin for 30 minutes at 37°C, the 28Srev and HEG ODNs being added followed by a further incubation period of 60 minutes, after which the efflux rate was monitored (figure 4.22) as described in section 2.5.2.1.

Figure 4.22 shows the efflux rate of the 28Srev and HEG ODNs in the presence and absence of monensin, the amount of ODN remaining associated with the cells is shown as a percentage of the original. Monensin appears to accelerate the rate of 28Srev and HEG efflux from cells. A classic efflux profile seen is a fast initial rate of ODN efflux. The efflux of the 28Srev ODN was increased by an average of 5.7% over 300 minutes when co-incubated with monensin, an average of 14.6% for the HEG conjugate compared to the control. The differing effect of monensin on efflux of the two ODNs indicates that they interact with the cells by different pathways.

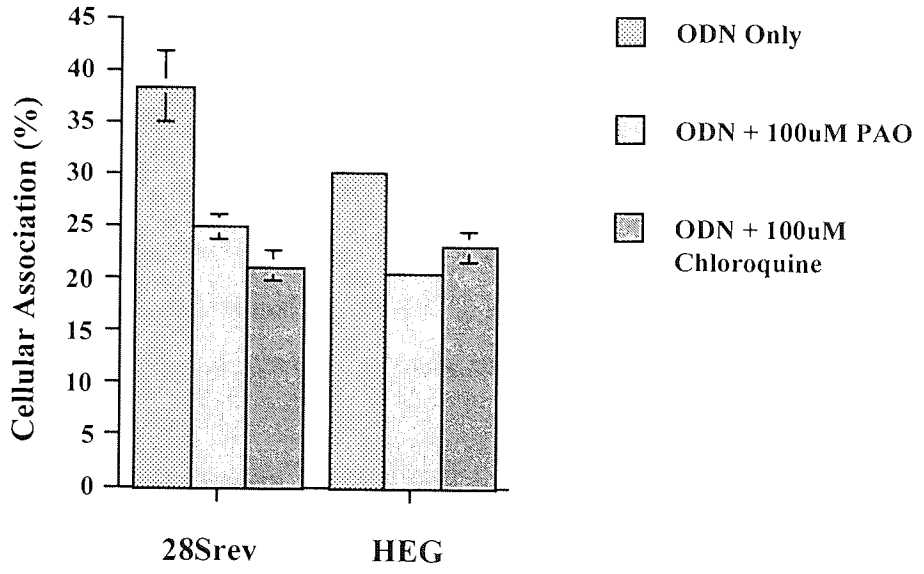
Figure 4.22: Effect of Monensin on Efflux of 28Srev Oligonucleotide and HEG Conjugate from RAW264.7 Cells. Graph to show the amount (%) of 28Srev and HEG ODNs effluxed from RAW264.7 cells following co-incubation of cells with monensin (10uM). Data is represented as the % ODN remaining cell associated at various time intervals following an initial 1hr incubation period at 37°C. n=4, bars=SD.



4.3.2.14 Effect of Endocytosis Disruption on 28Srev Oligonucleotide and HEG Conjugate

PAO and chloroquine are two other compounds used to disrupt the endocytosis mechanism, their modes of action are described in section 2.5.2.10. Due to time constraints, cellular association of only the control 28Srev and HEG ODNs was assessed with these compounds. Cells were pre-incubated for 5 minutes and 30 minutes in the presence and absence of PAO and chloroquine respectively as described in section 2.5.2.10, ODN was then added and cells incubated for a further 4 hours at 37°C (figure 4.23).

Figure 4.23: Effect of Phenyl Arsine Oxide and Chloroquine on the Cellular Association of 28Srev Oligonucleotide and HEG Conjugate. Graph to show the cellular association (%) of 28Srev and HEG ODNs with RAW264.7 cells in the presence and absence of the endocytosis disrupting agents Phenyl Arsine Oxide (PAO, 100uM) and chloroquine (100uM) following4 hours incubation at 37°C. n=4, bars=SD



Cellular association of both the 28Srev and HEG ODNs was reduced in the presence of PAO and chloroquine (figure 4.23). A 34.9% ($p<0.0001$) and 44.9% ($p<0.0001$) reduction in cellular association of 28Srev ODN were seen in the presence of 100μM PAO and 100μM chloroquine respectively in the RAW264.7 cells. The effect of these compounds on HEG cellular association was less pronounced than that of monensin, PAO and chloroquine causing reductions of 31.8% ($p<0.0001$) and 23.9% ($p<0.0001$) respectively.

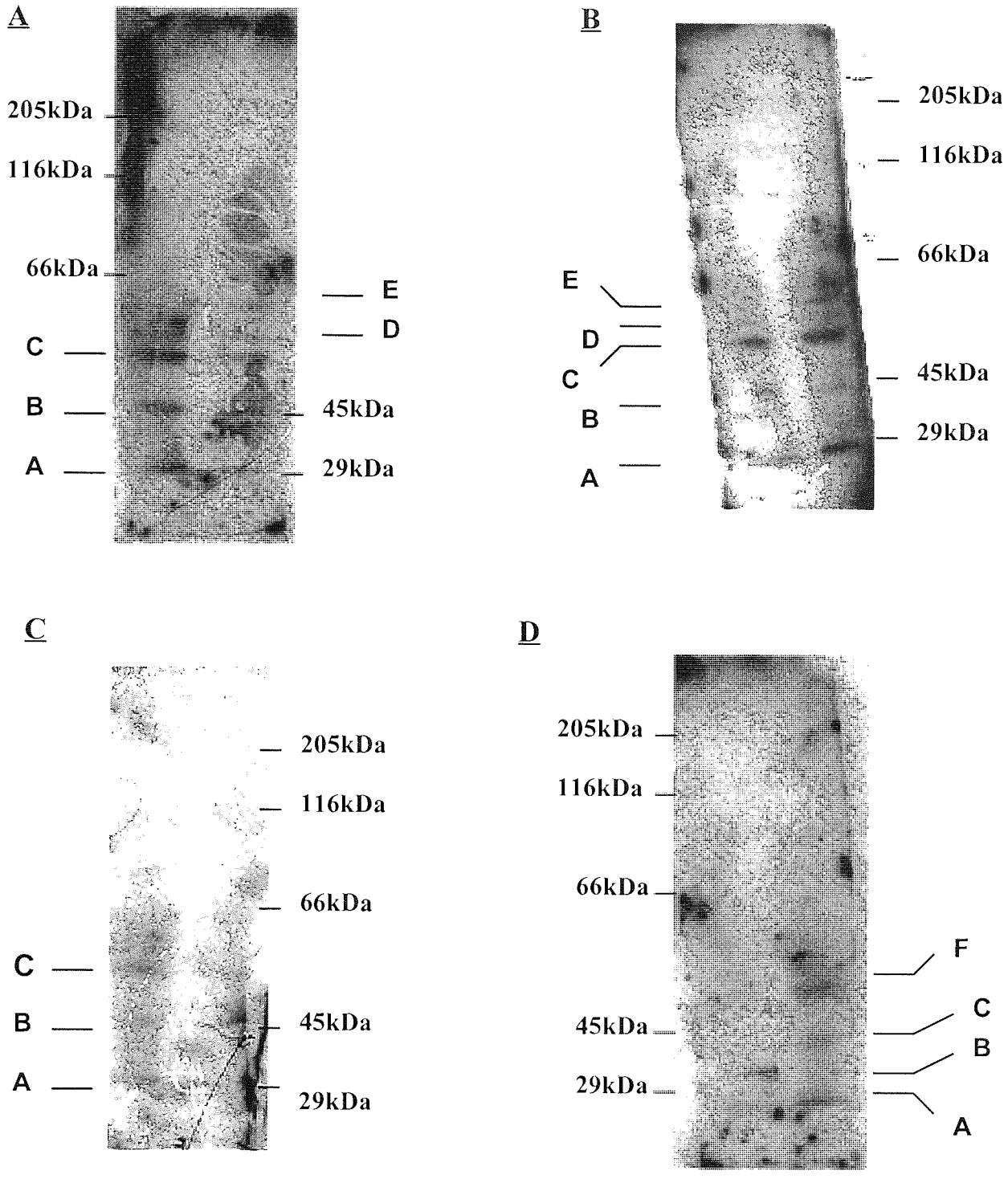
Both PAO and chloroquine reduce cellular association of the 28Srev and HEG ODNs, as does treatment with monensin, providing strong evidence for the involvement of an endocytic mechanism involving RME and receptor/binding molecule recycling back to the plasma membrane.

4.3.3 SOUTH-WESTERN DETECTION OF OLIGONUCLEOTIDE BINDING PROTEINS

All the cellular association data for the Srev ODNs indicates the presence of a receptor molecule involved in binding to RAW264.7 cells. To detect whether one or several proteins were involved in binding, RAW264.7 cell membranes were isolated (sections 4.2.5.1 and 4.2.5.2) and ^{32}P-labelled 28Srev, cholhex, C_{16} and HEG ODNs were incubated individually with the separated proteins which were immobilised on a nitrocellulose membrane (sections 4.2.5.3 and 4.2.5.4). Several researchers to detect putative binding proteins (Bennett *et al.* 1985; Akhtar *et al.* 1996) have used this approach, a modified protocol was used in these studies.

RAW264.7 cell membranes were isolated and the membrane proteins separated by electrophoresis alongside MW markers. Ponceau-S staining revealed an array of membrane proteins on the nitrocellulose membrane, over 15 separate bands were detected (data not shown). Autoradiography, following incubation of ^{32}P-labelled Srev ODNs with the nitrocellulose membrane with transferred proteins (Southwestern analysis,) are shown in figures 4.24A, 4.24B, 4.24C and 4.24D. Autoradiography film was exposed for 7 days.

Five bands were seen on the 28Srev autoradiograph, figure 4.24A, corresponding to approximately 29kDa (band A), 45kDa (band B), 55kDa (band C), 60kDa (band D) and 63kDa (band E). Other very faint bands were seen above the 66kDa marker on the original autoradiograph. This data illustrates the high degree of protein binding on the RAW264.7 cells, highlighting the non-specificity of this binding, which probably accounts for the high values of cellular association observed.



Figures 4.24 (A, B, C and D): Southwestern Analysis of RAW264.7 Cell Membrane Proteins Binding Oligonucleotides. Autoradiograph of RAW264.7 cell membrane proteins electrophoresed immobilised onto a nitrocellulose membrane probed with ^{32}P-labelled ODNs. Membrane incubation with (A) 28Srev ODN, (B) cholhex, (C) C_{16}, (D) HEG. Bands marked alphabetically denote protein-ODN complexes as detected by autoradiography.

Southwestern analysis of cholhex conjugate binding to RAW264.7 membrane proteins (figure 4.24B) revealed similar bands, detected for the 28Srev ODN. This conjugate therefore appeared to bind the same proteins as the unconjugated 28Srev, indicating that the ODN portion of the cholhex conjugate was responsible for protein association. Also, in common with the 28Srev ODN, other faint bands were detected, with their molecular weights between 45kDa and 66kDa.

The bands shown in figure 4.24C for the C_{16} conjugate Southwestern autoradiograph are very similar to the ones seen for 28Srev, three bands corresponding to molecular weights 29kDa, 45kDa and 55kDa (bands A, B and C respectively). However, in contrast to the 28Srev Southwestern, no other bands were apparent, indicating a higher specificity of binding. These data correlates with the cellular association data, the 28Srev bound RAW264.7 cells to a greater extent than the C_{16}.

HEG Southwestern analysis revealed bands corresponding to a protein of approximately 38kDa (figure 4.24D), in addition to the 29kDa, 45kDa and 55kDa proteins The HEG conjugate therefore bound a protein, which the 28Srev and other conjugates did not. Other bands were not seen on the autoradiograph, as was the case for the C_{16} conjugate, reflecting the lower non-specific cellular association of HEG compared to 28Srev and cholhex.

More than one protein was bound by each of the Srev ODNs. For all the Srev ODNs, three of these bands appeared to be the same protein for each ODN (29kDa, 45kDa and 55kDa). This experiment confirms that the Srev ODNs were binding RAW264.7 membrane proteins as indicated by the cellular association studies (section 4.3.2), no one specific protein being bound however.

4.3.4 CELLULAR ASSOCIATION OF HOMO-OLIGONUCLEOTIDES

The cellular association characteristics of ODNs composed of a single base type, homo-ODNs, were assessed in the RAW264.7 macrophages. The effect of length on cellular association was also evaluated, in RAW264.7 and A431 cells, in order to determine optimal ODN cellular association parameters. All the ODN used were phosphorothioates.

4.3.4.1 The Effect of ODN Base Composition

Phosphorothioate 7-mer homo-ODNs, 7A, 7C and 7T (section 4.2.5.1) association with RAW264.7 cells and 7G association with RAW264.7 and A431 cells was assessed after a 60 minute incubation period, the ODN were 5'-end labelled with ^{32}P-ATP. The effect of temperature on the cellular association of 7A and 7G ODN was also assessed at a 4°C incubation temperature after 60 minutes.

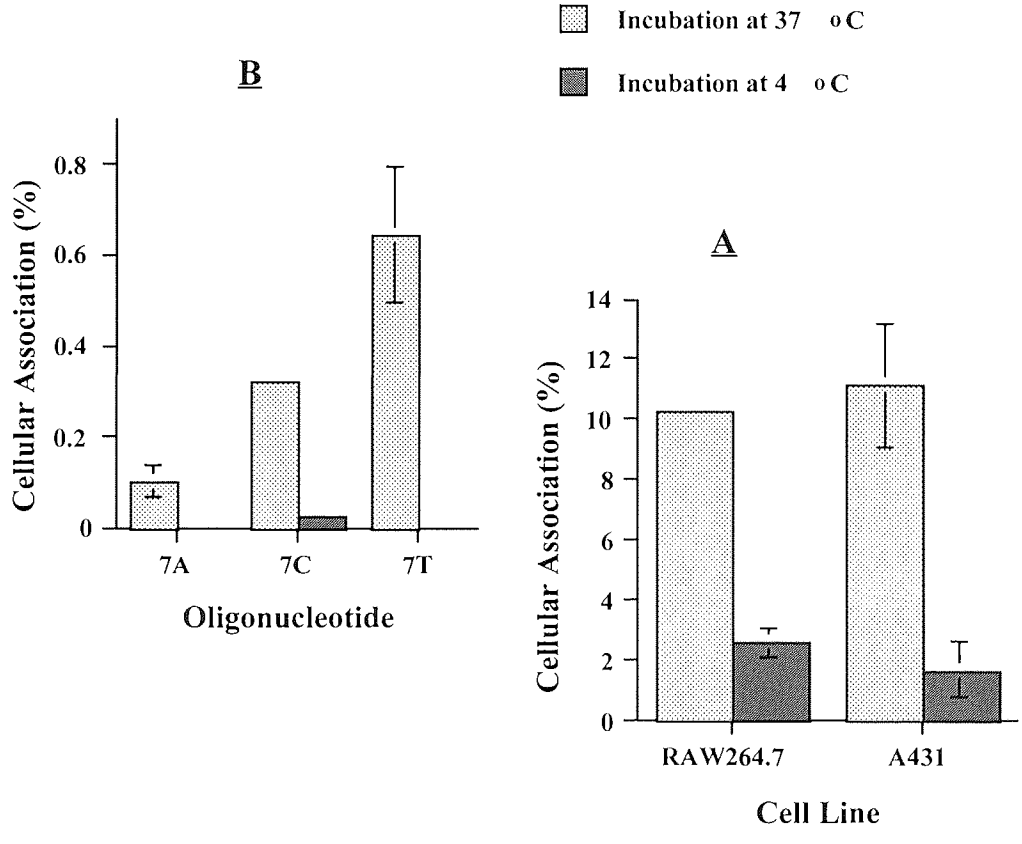
Cellular association of the homo-ODNs 7A, 7C and 7T to RAW264.7 cells is represented in figure 4.25A. The cellular association of the 7G ODN was significantly higher than the other homo-ODN, RAW264.7 and A431 cellular association of 7G shown in figure 4.25B.

The 7G ODN was found to have the highest cellular association, as has been reported by a number of other studies (Hughes *et al.* 1994; Peyman *et al.* 1995). 10% cellular association of the 7G sequence compared to values below 1% for the other base types in RAW264.7 cells was observed following 60minutes incubation at 37°C. The 7A ODN had the lowest cellular association to RAW264.7 cells, approximately 0.1% .

In RAW264.7 cells both 7G and 7C ODNs were also assessed for cellular association at 4°C and found to be temperature dependant (figures 4.25A and B). A431 cellular association of the 7G ODN was also found to be significantly higher at 37°C (figure 4.25A). The fact that incubation of ODNs at 4°C significantly reduces cellular association indicates the involvement of an active process involved in cellular binding.

The 7C and 7G ODNs may bind putative nucleic acid binding proteins on the cells (section 4.3.3), the association involving an active energy requiring process which may be inhibited at 4°C.

Figure 4.25 (A and B) : Cellular Association of 7-mer Homo-Oligonucleotides of Different Length. Graphs to show the cellular association (%) of 7T, 7A and 7C ODNs with RAW264.7 cells (A) and the cellular association of 7G ODN with both RAW264.7 and A431 cells, following 60 minutes incubation. n=4, bars=SD



4.3.4.2 The Effect of Oligonucleotide Length

The effect of ODN length on cellular association to RAW264.7 and A431 cells was assessed. Cells were incubated at 37°C for 60 minutes with homo-thymidine ODNs of different length, 7T, 14T, 20T, 28T and 36T phosphorothioates.

Figure 4.26: Cellullar Association of Homo-Thymidine Oligonucleotides of Different Lengths with RAW264.7 and A431 Cells. Graph to show the cellular association (pmol/well) of various homo-ODNs, 7T, 14T, 20T, 28T and 36T, with RAW264.7 and A431 cells, following 60 minutes incubation. n=4, bars=SD

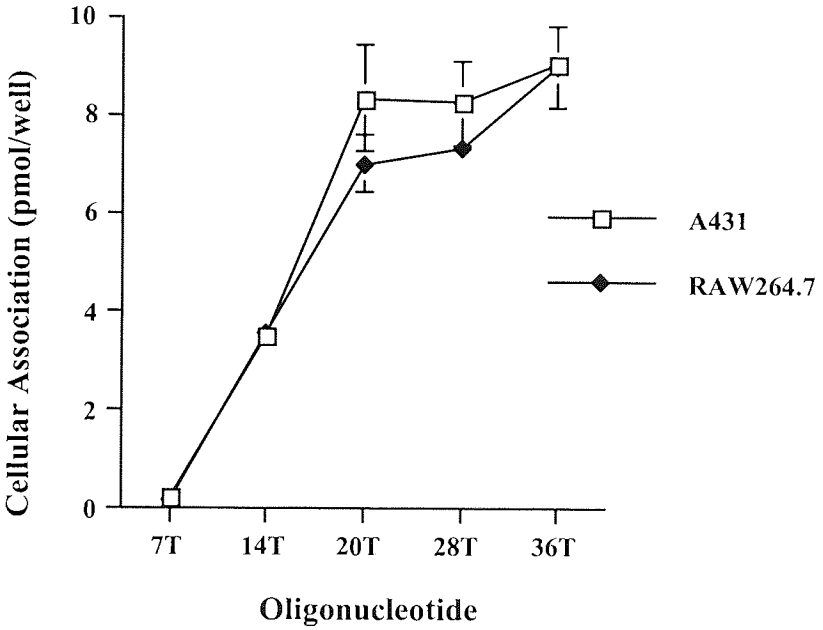


Figure 4.26 shows the cellular association profiles of the homo-thymidine ODNs in both RAW264.7 and A431 cells after 60 minutes incubation. Both cell lines bound the homo-thymidine ODN to very similar degrees (figure 4.26). Increasing ODN length from 7-mer to 20-mer gave rise to a sharp increase in cellular association in both cell lines, increasing length beyond 20 bases to a 28-mer and 36-mer however did not incite any further significant increase in cell association.

4.4 DISCUSSION AND CONCLUSIONS

The ODN sequence used was antisense to a region of the *rev* gene of HIV, the ultimate aim to inhibit HIV replication in the macrophages. Lipophilic moieties were conjugated onto the ODN in order to enhance uptake into cell and confer an increased stability to degradation by certain 5'-end nucleases. The binding of ODNs conjugated to three different lipophilic groups was assessed *in vitro* in the murine macrophage cell line RAW264.7 and human epithelial cell line A431. All the conjugates designed were synthesised by Prof. Tom Brown (Southampton University). Cellular association for each ODN conjugates (cholhex, C_{16} and HEG) was compared to an unconjugated ODN (28Srev). A ^{32}P-ATP label was conjugated to the 3'-end of the ODNs in order to monitor stability and binding to cells. Toxicity of the ODNs to the cells was not observed using the trypan blue test, any affects on cellular association were therefore attributed to an effect induced by the treatment.

A method described by Dagle *et al.* (1991) was used to assess the lipophilicity of the unconjugated 28Srev ODNs and conjugates (section 4.3.1.1). Dagle *et al.* (1991) report the partitioning of ODNs, modified with phosphoramidate internucleotide linkages (non-bridging oxygen replaced with an amine group), between an organic and aqueous phase. The organic phase was used to mimic the plasma membrane (Dagle *et al.* 1991), finding lipophilicity to increase as number of phosphoramidate bonds per ODN increased, HPLC analysis producing the same result, therefore demonstrating the validity of this method for determining lipophilicity of ODNs. This method confirmed the increased lipophilicity of cholhex, C_{16} and HEG conjugates compared to control unconjugated 28Srev ODN. An increase in lipophilicity achieved by these moieties should therefore enhance ODN binding to the plasma membrane.

All the ODNs were found to be resistant to degradation for up to 3 hours incubation at 37°C with RAW264.7 cells or the epithelial cell line A431 (section 4.3.2.1). However, 5 hours incubation with RAW264.7 cells, the C_{16} conjugate was almost totally degraded into shorter fragments and the HEG conjugate also showed some degradation. Therefore, studies with the C_{16} and HEG conjugates were not extended beyond a period of 3 hours in RAW264.7 cells. This degradation of the C_{16} and HEG conjugates was not observed following 5 hours incubation with A431 cells.

The instability of the C_{16} and HEG conjugates in RAW264.7 cell cultures may render them inappropriate for therapeutic use if uptake into cells requires longer than 3 hours exposure. The increased degradation rate of the C_{16} and HEG conjugates, compared to the unconjugated 28Srev ODN is theoretically unexpected. Protecting the 5'-end of the ODN should be conferred by conjugation of the lipophilic moiety to 5'-end nucleases. However, by some unknown mechanism, degradation was elevated in the culture medium by C_{16} and HEG moieties. Both the 28Srev ODN and cholhex conjugate were shown to be stable after 5 hours incubation with RAW264.7 or A431 cells. The 28Srev ODN and cholhex conjugate appear to be good candidates for an antisense-based drug due to their stability. *In vivo* studies need to be performed in order to evaluate stability to degradation by cellular nucleases.

A cholesterol moiety was found to enhance ODN binding to RAW264.7 cells, as determined by fluorescence microscopy of cells incubated with a fluorescein labelled ODN-cholesterol conjugate (section 4.3.2.2). A punctate distribution of fluorescence was seen in cells incubated with this conjugate, indicating intravesicular localisation. The control ODN bound to a lesser extent to cells observed as a lower degree of fluorescence, in a more dispersed pattern. Bennett *et al.* (1992) and Shoji *et el.* (1996) found fluorescently labelled phosphorothioate ODNs distributed in a punctate pattern, attributed to encapsulation within endosomal or lysosomal vesicles in the cytoplasm. Noonberg *et al.* (1993) reported a similar distribution of phosphodiester ODNs in cultured keratinocytes, demonstrating enhanced of ODN binding as a result of cholesterol conjugation.

Three different cell lines (RAW264.7 macrophages, A431 epithelial cells and U87-MG glioma cells) were assessed for their binding of the 28Srev ODN and conjugates, in order to determine whether these lipophilic groups enhanced binding in a cell dependant manner. The cellular association of the 28Srev ODN and cholhex conjugate was highest in RAW264.7 cells, the C_{16} and HEG conjugates binding to all three cell lines to similar degrees, approximately 50% that seen for 28Srev and cholhex. Noonberg *et al.* (1993) state that the quantity and mechanism by which ODNs are internalised into cells differs between cell lines in their study, primary cultured keratinocytes internalised a greater amount of ODN compared to various other cell lines including an immortalised keratinocyte cell line. The intracellular distribution of ODNs was found to follow the same general trend in five cell lines studied by Temsamani *et al.* (1994), however the actual quantities of ODN differing between cell lines. Predicting the association profile of an ODN with cells in culture is difficult due to the many factors, which differ from one cell line to another. The size of cells is crucial when making such comparisons, the RAW264.7 macrophage cells are considerably smaller than the A431 and U87-MG cells, which is reflected by the seeding densities in the 24-well plates, highlighting the need for taking factors such as cell size into account. The surface area over which cell association occurs is particularly important when passive and active endocytosis mechanisms are compared. One of the ways to account for size is to normalise cells to protein content. Overall, the C_{16} and HEG conjugates had no selective affinity for one cell line and did not enhance binding of the 28Srev ODN. Attempts at producing a shorter HEG-ODN conjugate proved unsuccessful.

The unconjugated 28Srev ODN had an unusually high cellular association to RAW264.7 cells, equivalent to approximately 25% of the total added, after 60 minutes incubation at 37°C. This is significantly higher than the C_{16} and HEG conjugates. The high percentage cellular association may be attributable to a combination of factors. The antisense *rev* sequence has high 'G' content, 'G' bases having a greater tendency for cellular association than other bases (Hughes *et al.* 1994). Both RAW264.7 and A431 cells bound 7G ODNs to a greater degree than other homo-nucleotides (section 4.3.4.1), in agreement with Hughes *et al.* (1994), thus supporting the theory of high

cellular association of G-rich ODNs. The $^{5'}$CCGC$^{3'}$ base sequence of the 28Srev ODN may induce the formation of a secondary structure which enhances binding, similar to G-quartet loop structures (Peyman *et al.* 1995) and tetraloops of CCCG quartets in 30-mer ODNs (Van Dongen *et al.* 1996). The nonsense sequence synthesised, which also possessed the $^{5'}$CCGC$^{3'}$ sequence, was found to have a similar degree of cellular association (section 4.3.2.4). The greater capacity of the antisense 28Srev sequence for cellular association may therefore be the result of a combination of factors; the $^{5'}$CCGC$^{3'}$ sequence, the length of the ODN and the phosphorothioate backbone chemistry of the ODN, all contributing to non-specific protein binding on the cell surface. The C_{16} and HEG conjugated moieties may possibly hinder the natural tendency of this ODN to bind strongly to cells.

Cellular association for all the Srev ODNs, with RAW264.7 cells, was found to be temperature dependant (section 4.3.2.5). The ODN binding profiles differed significantly from that of the fluid phase marker mannitol, indicating that different mechanisms were operating. The cellular association profiles observed for the Srev ODNs at 4°C are indicative of a passive process such as fluid phase endocytosis as described by Stein *et al.* (1993) and Nakai *et al.* (1996). Kreig *et al.* (1993) reported a lower degree of cell association for a cholesterol-conjugated phosphodiester ODN at 4°C compared to 37°C. They observed extracellular surface association at 4°C, with cytoplasmic and little nuclear localisation at 37°C. The cellular association for the cholhex and HEG conjugates increased in a linear manner with time. Association of the control 28Srev plateaued at around 5 hours. The C_{16} conjugate cellular association data was inaccurate at longer incubation periods due to significant degradation. A mechanism other than fluid phase endocytosis is implicated by these results as temperature dependency indicates an energy requiring process.

The slow efflux of ODNs from cells indicates high affinity association to intracellular organelles, possibly sequestration in compartments deep within the cell (Tonkinson *et al.* 1994). Molecules internalised via a fluid phase mechanism would be more readily effluxed from cells, as suggested by Besterman *et al.* (1981). The efflux pattern for mannitol is representative of molecules internalised into cells via a passive fluid phase

mechanism, efflux of other fluid phase markers such as sucrose are found to be in equilibrium with endocytosis (Besterman *et al.* 1981). Efflux profiles for the Srev ODNs in both macrophages and A431 cells were slowest for cholhex in both cell lines (section 4.3.2.5), possibly sequestered in deep compartments according to the theory of Tonkinson *et al.* (1994). In RAW264.7 cells mannitol and the 28Drev ODN were effluxed at the fastest rate implying an alternative mechanism for binding and/or sequestration into shallow compartments within the cell. In comparison to the 28Srev ODN in RAW264.7 cells, efflux for HEG was slower and that of the C_{16} conjugate faster. Therefore the C_{16} appeared neither to enhance Srev ODN binding nor slow down its efflux. The efflux profiles for all the Srev ODNs differed for the two cell lines, again giving an indication of variance between cell lines. Temsamani (1981) found uptake and efflux in dynamic equilibrium. Tonkinson *et al.* (1994) revealed phosphodiesters were effluxed faster than phosphorothioates, although data cannot be extrapolated to other cell lines, as the nature of efflux is cell type specific. Tonkinson *et al.* (1994) also reported trafficking of PO- and PS-ODNs by different mechanisms in HL60 cells. Tonkinson *et al.* (1994) state that PS-ODNs enter acidic compartments whereas PO-ODNs do not.

Gao *et al.* (1992) proposes a two-compartment mechanism for efflux of PS-ODNs, seen as a bi-phasic profile. An initial burst of release during the first 60 minutes was observed, followed by a slow release phase (50% remaining intracellularly after 8 hrs). An early endosome-like intracellular compartment is proposed to be involved in efflux, deeper compartments sequestering the remaining ODN. The efflux data for the Srev ODNs, in RAW264.7 and A431 cells, had a similar bi-phasic profile, with an initial rapid release seen during the first 60 minutes followed a reduced rate of efflux.

NaN_3 and 2DG are commonly used as metabolic inhibitors to investigate ODN binding (Loke *et al.* 1989; Gao *et al.* 1992; Wu Pong *et al.* 1994a and 1994b; Shoji *et al.* 1996). These inhibitors were found to inhibit cellular association of all the Srev ODNs by an average of 35%, complete inhibition was however not attained (section 4.3.2.6). The same observation was made for the mono-mannose conjugate (section 3.2.6), with cellular association not completely depleted by these inhibitors. The ODN

fraction, which bound cells even in the presence of metabolic inhibitors, must bind via a energy independent interaction, possibly a ionic interaction between the ODN and cell surface proteins, or be internalised by a passive mechanism. An alternative interpretation is that the pre-incubation period of the cells with the inhibitors was not adequate to completely deplete cellular energy levels. Wu Pong *et al.* (1992) attributes this low level of cellular association to energy production in the presence of the inhibitors, enabling partial association. As all the ODNs were inhibited to the same degree by NaN_3 and 2-DG, we can infer that association of no single ODN was more energy dependant than the others, despite the differing degrees of cellular association. The significant reduction in cellular association of the ODNs implies the involvement of an energy dependant binding and uptake process.

The Pronase studies (section 4.3.2.7) provided evidence for the involvement of cell surface proteins, on RAW264.7 cells, in binding the Srev ODNs. The cholhex and HEG conjugates were found to be least affected by the action of Pronase. Therefore, we can assume that a significant fraction of the cholhex and HEG conjugates were internalised or bound to non-pronase sensitive components of the cell surface. The combination of slow efflux rate and a reduced Pronase sensitive- association for cholhex, combines with a high cellular association is high indicates a high degree of internalisation. The HEG conjugate had a lower cellular association than the control 28Srev (section 4.3.2.5). This associated fraction was less Pronase sensitive than the 28Srev (section 4.3.2.7) and the amount of HEG effluxed out of the cell also lower, indicating that although less was bound by the macrophages, this fraction was more tightly bound and possibly internalised. Cellular association of the C_{16} conjugate was most affected by Pronase treatment, indicating the highest degree of surface protein binding and therefore the lowest degree of internalisation. The 28Drev ODN was also significantly affected by Pronase treatment. The Pronase experiments suggest ODN binding to cell surface proteins. Many studies report cell surface proteins, which bind and internalise ODNs. Bennett *et al.* (1985) identified a 30kDa DNA binding protein on the surface of white blood cells, Loke *et al.* (1989) isolated an 80kDa binding protein on HL60 cells and Beltinger *et al.* (1994) identified five binding proteins ranging between 20kDa to 143kDa. Wu Pong *et al.* (1994a and 1994b) showed PO-

ODN uptake in erythroleukeamic cells to be reduced by trypsin treatment, the reduction attributed to an ODN binding protein on the cell surface similar to reductions in cellular association of the Srev ODNs by Pronase treatment. Beck *et al.* (1996) demonstrated 87% reduction in cellular association of phosphodiesters following Pronase treatment (at 4°C), the fraction not affected by Pronase was attributed to either an internalised fraction, a non-protein bound or tightly bound fraction. 50% of PS-ODNs were removed by Pronase treatment at 4°C in comparison (Beck *et al.* 1996).

All the Srev ODNs were affected by self-competition, indicating a limited binding capacity, possibly attributable to specific binding sites on RAW264.7 cells. Similar self-competition data is reported by Beck *et al.* (1996), for a 20-mer PS-ODN in intestinal epithelial cells, reduction in cellular association in the presence of competition attributed to a putative cell surface binding mechanism. Cellular association of the 28Srev ODN was reduced to the greatest degree, indicating the most limited binding capacity on the cells or binding with a low affinity. The cholhex and C_{16} conjugates were most affected by self-competition, indicating the fewest number of putative binding sites on the surface or high affinity binding. The HEG conjugate was least affected by self-competition indicating a high binding capacity. Beltinger *et al.* (1995) found a decrease in binding of a PS-ODN in the presence of excess unlabeled PS-ODN, these self competition studies combined with Scatchard plot analysis were used to predict the number of binding sites on the surface of human leukaemia cells.

The role of the 28Srev ODN component in cellular association of the conjugates was investigated (section 4.3.2.9). Competition of the conjugates with excess 28Srev ODN resulted in no significant reduction in cellular association. Therefore the conjugate and 28Srev ODN components of the complex appeared to bind to different sites on the cell. Therefore the lipophilic moiety of the ODN-conjugate appeared to be involved in binding to RAW264.7 cells. It may be that the 28Srev ODN and conjugate moiety, with the latter having a greater affinity bound the same site.

The reason for increased HEG conjugate binding in the presence of excess 28Srev ODN was not established, although the increase was significant. The effect of a range of different 28Srev ODN concentrations on HEG conjugate cellular association needs to be evaluated, determining whether there may be a point where the 28Srev aids HEG binding.

To fully understand the binding specificity for each conjugate, various competition studies need to be performed. If the cellular association of each conjugate were assessed in the presence of an excess of different conjugate the data would provide information on affinity of the cells for binding a conjugate and whether identical or adjacent sites on the cell surface were occupied. Binding to adjacent sites may cause steric hindrance to binding at neighbouring sites.

Dextran sulphate and heparin were found to bind the basic-fibroblast growth factor receptor protein with a high affinity (Kajio *et al* 1992; Guvakova *et al.* 1995), highlighting the tendency of these polyanions to interact with cell surface proteins. Bennett *et al.* (1985) used heparin to compete with bacteriophage DNA for putative binding sites on the surface of white blood cell membranes, a decrease in DNA binding resulting from competition. Competition of the ODNs with the non-nucleic acid polyanionic molecules, dextran sulphate, resulted in significantly reduced cellular association for the 28Srev ODN, C_{16} and HEG, but not the cholhex conjugate (section 4.3.2.10). This indicated that binding of these ODNs to macrophages was not specific but due to an ionic interaction between the anionic ODN and cationic cell surface proteins. Cholhex appeared to bind more specifically, however, as polyanions did not affect binding. Dextran sulphate was the only polyanion with an inhibitory affect on 28Srev and HEG ODN cellular association, which indicates an ionic interaction between these ODNs and the cell. However, an ionic interaction may not be the sole factor in binding, because heparin did not affect binding. The C_{16} conjugate was most affected by dextran sulphate, which implies that ionic interactions play a major role in binding the macrophage cell surface. These studies are similar to Wu Pong *et al.* (1992) who found dextran sulphate to decrease uptake of a 21-mer PO-ODN by 48% and attributed this to interference with the charge-charge interaction between the ODN

and the cell surface. Hawley *et al.* (1996) found heparin to compete with a PS-ODN for binding to a human epithelial cell line. Further competition studies involving different concentrations of competitor and different types of competitor need to be performed to establish whether the bonding is ionic.

To further investigate the nature of ODN conjugate interactions with the cell surface, the effect of pH on cellular association was investigated (section 4.3.2.11). As the pH of culture medium decreased, the cellular association of all the ODNs increased. A decrease in pH renders proteins more cationic and hence increases their attraction to the polyanionic ODNs. If the changes in cellular association observed during pH change is to be used as a measure of protein binding, the unconjugated 28Srev was protein bound to the greatest degree and the C_{16} conjugate the least, the order being 28Srev > HEG > cholhex > C_{16}. Goodarzi *et al.* (1991) found the efficiency of ODN binding to eukaryotic cell membranes increased under acidic conditions, a 34kDa binding protein was identified, consistent with Bennett *et al.* (1985), Loke *et al.* (1989), all identifying proteins which show competitive inhibition of binding in the presence of 50-fold ODN excess. Kitajima *et al.* (1992), found a 10-fold increase in cellular association of PS-ODN when cells were incubated at pH4.5, two cell membrane binding proteins, which bound the ODN at acidic pH and could be competitively inhibited by ODNs of the same and different sequence. Beck *et al.* (1996) attribute the increased cellular association of PO-ODN and PS-ODNs at lower pH to an increase in association with cell surface proteins.

Competition between the Srev ODNs and dextran sulphate (section 4.3.2.10) revealed cellular association of the C_{16} to be little affected, which indicated a low degree of binding to proteins via ionic interactions. The pH data also indicates that the C_{16} was interacting with surface proteins to a lower degree than the other ODNs. Cellular association of the cholhex conjugate was not affected by the presence of dextran sulphate, however, a decrease in pH resulted in greater cellular association indicating more proteins were bound. Therefore, although cholhex may be binding surface proteins, the interactions involved may be too strong to be competed for by this polyanion.

To verify that the increase in cellular association was due to protein binding as a result of a decrease in pH, Pronase was used to remove protein associated ODNs at pH7.0 and pH5.0 (section 4.3.2.11). The effect of Pronase on cellular association of 28Srev ODN and HEG conjugate at this pH revealed that more ODN was stripped off at the lower pH.

Monensin is a sodium ionophore, which is, diffuses across the membrane (therefore lipid soluble), it is able to prevent the processing of molecules from the golgi, such as cholesterol transport to the mitochondria. Monensin was used to investigate the Srev ODN uptake mechanism in RAW264.7 cells (section 4.3.2.12). One affect of monensin is to neutralise intracellular acidic compartments by collapsing Na^+/H^+ gradients. RME involves the internalisation of a receptor/ligand complex into acidic intracellular vesicles, such as endosomes and lysosomes, in which the complex dissociates releasing the ligand and free receptor. The receptor in some cases recycles back to the surface. Therefore, monensin may cause a reduction in cellular association via inhibition of receptor recycling because the receptor/ligand complex does not dissociate. Cellular association of all the Srev ODNs was significantly reduced in the presence of monensin. This indicates that all the ODNs are trafficked within the cell via a mechanism involving acidic compartments, which were neutralised by monensin (section 4.3.2.12.1). This depletion in cellular association could be attributed to pH neutralisation of endosomes or lysosomes by monensin. Surface receptors may fail to recycle back to the surface because the receptor/ligand complex did not dissociate, therefore further ligand binding at the surface reduced. As monensin has such an array of affects on the cell it is not conclusive that neutralisation of intracellular compartments was causing the decrease in receptor recycling and subsequent reduction in cellular association.

As monensin affects later stages in endocytosis, such as receptor recycling and function of the golgi, in the experiments described above cells were pre-incubated with monensin prior to ODN addition. Mollenhauer *et al.* (1990) state monensin can exert an affect 2-3 minutes after exposure, most studies pre-incubating cells with

monensin for 15-30 minutes prior to ligand addition (Stein *et al.* 1984; Hertel *et al.* 1985; Wu Pong *et al.* 1992 and 1994a). In order to confirm that monensin was acting at an early stage in the binding and uptake process (section 4.3.2.12.2), the pre-incubation stage was omitted and cells co-incubated with monensin. Binding of the 28Srev ODN and HEG conjugate to RAW264.7 cells revealed that cellular association was affected by monensin, at a very early stage (section 4.3.2.12). This outcome can be attributed to one of three explanations; either these ODNs may enter the cell and engage in the endocytosis cycle very rapidly, accounting for monensin having an affect, or that cell surface binding was impeded, or monensin accelerated efflux.

Investigating the efflux rates for the 28Srev and HEG, in the presence of monensin, revealed a significant increase in the loss of these ODNs from RAW264.7 cells. Therefore monensin appears to accelerate efflux of ODNs. An increase in efflux is unexpected, if 28Srev and HEG are internalised via a RME mechanism. If a receptor were involved, and trapped within acidic intracellular vesicles, on monensin treatment then this should be reflected in an inhibition of efflux. Some aspects of golgi apparatus trafficking, which are also inhibited by monensin would also inhibit efflux due to entrapment of the ODN. Therefore, the mechanism involved in cellular association and intracellular trafficking of the 28Srev and HEG ODNs, involves a more complex system than just internalisation into acidic vesicles or golgi apparatus. It would be interesting to assess the other two conjugates in the same way as the 28Srev and HEG to determine if the same occurs with respect to efflux following monensin treatment. Monensin may affect the trafficking of vesicles to endosomes and lysosomes, therefore these vesicles may be immediately effluxed out of the cell thus accounting the increase in efflux rate.

PAO and chloroquine, agents that disrupt endocytosis, were used to investigate RAW264.7 cell uptake of the 28Srev and HEG (section 4.3.2.13). Chloroquine is a weak base which increases lysosomal pH (Stein *et al.* 1984; Teitelbaum *et al.* 1986), like monensin, disrupting acidic intracellular compartments by neutralising them. Inhibition of receptor/ligand dissociation due to neutralisation of the pH of acidic intracellular compartments was concluded by Teitelbaum *et al.* (1986), cells treated

with monensin and chloroquine showing a reduction in surface receptor number and therefore ligand association. Hertel *et al.* (1985) used PAO to inhibit the endocytosis of EGF and β-adrenergic receptors. Nakai *et al.* (1996) found cellular internalisation to be inhibited by PAO, concluding that binding sites on the cell membrane mediated uptake by a mechanism involving endocytosis. Both agents were found to significantly reduce cellular association of these ODNs, supporting the theory of a RME mechanism in binding and internalisation.

The investigations performed on Srev ODN binding, in this thesis, implicate a cell surface binding protein on the surface of the RAW264.7 cells. We have demonstrated a binding profile differing from that of a fluid phase marker, temperature dependency, inhibition by metabolic inhibitors, reduction in cellular association by Pronase treatment, self-competition, competition with other polyanions, an increased binding at low pH and the effects of agents that negatively disrupt endocytosis. Southwestern analysis of macrophage cell membranes revealed several proteins involved in binding the Srev ODNs (section 4.3.3). Three common proteins, of 29kDa, 45kDa and 55kDa in size, were bound by the 28Srev ODN and conjugates. In addition the 28Srev and cholhex bound 60kDa and 66kDa proteins. The cholhex conjugate also bound a number of other proteins with a lower affinity indicating a very high degree of protein association. The HEG conjugate also bound an additional protein of 38kDa. The C_{16} conjugate, which had the lowest degree of cellular association, only bound the three proteins detected for all the ODNs. This data is consistent with the theory of cell surface binding proteins on the RAW264.7 plasma membrane. Time constraints did not allow further analysis of these proteins. It is not suprising that similar proteins appear to be involved in association of these Srev ODNs due to the fact that the conjugate molecules share a large consensus proportion, the 28Srev ODN moiety. The degree to which each of these conjugates associates with these proteins will be influenced by the nature of their conjugate moiety.

Bennett *et al.* (1985) detected a 30kDa DNA binding protein in the membranes of neutrophils, monocytes and lymphocytes, using the same method described here, this

may correspond to the 29kDa protein detected by the Southwestern studies. Several ODN binding proteins were also detected by Akhtar *et al.* (1996) on an epithelial carcinoma cell line. A 46kDa protein had the greatest degree of PO-ODN and PS-ODN binding, while other proteins also bound PS-ODNs, and this was more apparent at lower pH. The 46kDa-protein bound in these studies may be the same as the 45kDa protein seen in Southwestern analysis of the Srev ODNs binding RAW264.7 cell membranes. Hawley *et al.* (1996) also used the Southwestern technique to show T15 fibroblasts, monocytes and lung carcinoma cell lines to possess a protein of approximately 46kDa that binds ODNs, although binding to other proteins was also detected. Hawley *et al.* (1996) show that ODN binding to a 46kDa protein is competed by excess ODN and heparin, suggesting a degree of non-specificity for binding.

Of the three conjugates assessed, only the cholhex conjugate showed enhanced cellular association to RAW264.7 macrophages *in vitro* compared to the unconjugated 28Srev. However, this was only marginal, C_{16} and HEG conjugate actually reduced binding. RAW264.7 cell surface proteins were identified which bound these ODNs. This data appears to indicate a RME mechanism. The C_{16} and HEG conjugates probably impede ODN binding possibly due to steric hindrance caused by the conjugate moiety. Therefore the conjugation of lipophilic moieties onto ODNs will not necessarily enhance binding, other factors have to be taken into account such as size of the moiety. The 28Srev ODN and cholhex conjugate appear to be good candidates for an antisense molecule, C_{16} and HEG conjugates proving to be poor.

Cellular association assessment of ODNs of differing base composition revealed RAW264.7 cells to bind homo-ODN to differing affinities, in the order 7G>7T>7C>7A (section 4.3.4.1). The high degree of cellular association of the 7G ODN may be a characteristic of cells *in vitro*, both RAW264.7 cells and A431 cells binding the 7G to a similar degree. Hughes *et al.* (1994) found the same order of preference for cellular association in Chinese Hamster ovary tumour cells, homo-ODN (10-mer phosphorothioates) bound in the order G>T>C=A, this order was maintained when comparing binding to liposome-encapsulated ODNs. This study suggests that

the differences in uptake of homo-ODN may be a function of both differing associations to cell surface molecules and efflux mechanisms.

An optimal ODN length at which there is a high degree of cellular association was found when homo-thymidine ODNs were assessed. Beyond a 20-mer ODN length, in both RAW264.7 and A431 cells, the cellular association profiles began to plateau, further increases in length not inciting any significant increase in binding (section 4.3.4.2). Therefore it appears that the use of an antisense ODN beyond a 20-mer in length in order to increase cellular association would be unnecessary, and only incite an increase in the likelihood of non-specific binding to a target sequence.

The above data determining the cellular association characteristics of the different nucleotide bases and ODN length have produced two important conclusions which can be applied to ODN design. Firstly ODN rich in guanosine and thymidine bases will have a higher cellular association than those rich in cytidine and adenosine bases. Secondly ODN 20-mer in length will have optimal cellular association. These conclusions have only been reached from the data obtained from two cell lines, therefore cannot be applied to all cells. However, the data obtained from two very different cell lines implies that cell lines may generally have the same parameters for binding ODNs.

5.1 INTRODUCTION

Effective ODN therapy is based on these molecules gaining entry into the cytoplasm and remaining stable for long enough to reach the target site. Extracellular stability and delivery of ODNs into cells has previously been discussed and the various methods that have been employed to circumvent the associated problems (sections 1.5 and 1.9). Once ODNs have gained entry into the cell intracellular stability and trafficking issues must be addressed.

5.1.1 FATE OF INTERNALISED OLIGONUCLEOTIDES

The intracellular fate of ODNs is dependent on the mechanism employed for their entry. Very little is known about ODN trafficking inside the cell. Whether entry occurs via fluid phase endocytosis or a receptor mediated mechanism, ODNs become encapsulated into intracellular vesicles, primarily endosomes followed by lysosomes (Alberts *et al.* 1989), a diagrammatic representation of events is shown in figure 1.5.

5.1.1.1 Pre-Lysosomal Compartments

The intracellular vesicles that initially contain molecules ingested by fluid phase endocytosis or RME have a very short life span, they are rapidly transferred to intermediate membrane bound organelles called endosomes. At least three types of endosomes exist, found in different regions intracellularly. Peripheral endosomes are found near to the plasma membrane, peri-nuclear endosomes and endo-lysosomes in a more central area of the cytoplasm (Alberts *et al.* 1989).

Endolysosomes have a pH of between 5-6, due to their membrane possessing an ATP-driven proton pump. It is thought that endolysosomes are the site where ligand

degradation begins. Newly synthesised enzymes from golgi apparatus are transported to endolysosomes. The slightly acidic endosomal pH plays an important role in ligand-receptor dissociation, conformational changes occurring as a result of the pH change and causing ligand release and receptor recycling back to the plasma membrane or degradation. Eventually endolysosomes fuse into or are converted into lysosomes. The process by which the contents of endolysosomes are moved to lysosomes is unclear; fusion of adjacent compartments is one possible explanation or compartment maturation. Unless the contents of endosomes are specifically retrieved, they are degraded in lysosomes (Alberts *et al.* 1989). Insulin is a ligand, which is internalised by RME, a small fraction of the insulin-receptor complex is trafficked to the golgi apparatus and nucleus intact, the remaining fraction degraded in lysosomes (Goldstein *et al.* 1979). The transferrin receptor is an example of a receptor, which is trafficked back to the cell surface following RME of its ligand (Alberts *et al.* 1989).

5.1.1.2 Lysosomes

Lysosomes are intracellular compartments, which contain acid hydrolytic enzymes whose function is the controlled digestion of macromolecules, whether they are intra-cellular or extra-cellular. Many hydrolytic enzymes are found within lysosomes; proteases, nucleases, phosphatases, phospholipases, lipases and sulphatases are some of the many (Duncan *et al.* 1980). Intra-lysosomal pH is approximately 5.0, the pH at which the hydrolytic enzymes are optimally active. The pH is maintained in the lysosomes by an ATP-driven proton pump in the lysosomal membrane, the membrane itself is protected from degradation by its contents due to highly glycosylated proteins (Alberts *et al.* 1989).

Phase contrast and electron microscopy and endocytic tracers have enabled lysosomal compartments to be identified. Lysosomes are distinct from other intracellular compartments, the size, shape and volume of lysosomes vary depending on cell type, environment and endocytic load (Steinman *et al.* 1983). The isolation of lysosomes is classically achieved by cell fractionation, because their density can be made sufficiently different from other organelles by the addition of surfactants, such as

Triton, to enable gradient separation (Trouet *et al.* 1974; Steinman *et al.* 1983). Steinman *et al.* (1983) review the studies carried out to deduce lysosomal involvement in endocytosis and membrane fusion. The latter thought is to be a key event on trafficking of endocytosed molecules. Study of lysosomal function has involved the use of staining materials such as acridine orange or electron dense colloids, thereby allowing fusion events between intra-cellular vesicles to be studied. Low temperatures (<18°C) were found to decrease the rate of lysosomal membrane fusion and the ligand was found to accumulate in the pre-lysosomal vesicle endosomes. Polyanions, such as dextran sulphate, were also found to retard fusion rates.

Lysosomes have a vital role within the cell, they are not only involved in the digestion of intra- and inter-cellular debris and phagocytosed micro-organisms, but also in cell nutrition. For example, the release of free cholesterol from low-density-lipo-proteins due to hydrolytic enzymatic action occurs within lysosomes (Steinman *et al.* 1983). A role for lysosomes in the degradation of cytoplasmic RNA has also been suggested.

Molecules can be trafficked to lysosomes by one of three pathways. Autophagy occurs whereby intracellular molecules, which are obsolete parts of the cell itself, are transported to lysosomes for degradation, phagocytosis whereby extracellular molecules or micro-organisms are ingested by the cell via engulfment and internalisation into a vesicle, and endocytosis, the invagination of plasma membrane and extracellular material into a intracellular vesicle. In all cases the intracellular compartments formed eventually terminate in lysosomes where digestion occurs (Steinman *et al.* 1983; Alberts *et al.* 1989).

5.1.2 OLIGONUCLEOTIDES AND LYSOSOMAL DEGRADATION

Whether ODNs gain entry into the cell by a receptor mediated or fluid phase endocytosis mechanism the intra-cellular site of localisation will most commonly be the lysosome, (figure 1.5). Sequestration within these compartments implies that ODNs will not be able to reach their target and will be enzymatically degraded.

Amongst the many enzymes within lysosomes are nucleases, esterases and phosphatases, key enzymes that degrade ODNs. The phosphatases work by a mechanism involving de-phosphorylating the terminal phosphate groups (Shaw *et al.* 1991). Nucleases are a group of enzymes, which degrade both DNA and RNA at the phosphodiester bond between nucleotides. The degradation of nucleic acids to their constituent nucleosides by the enzymatic activities of lysosomes has been shown in a number of studies. The mechanism by which degradation products are released from lysosomes is less well understood, specific transport mechanisms are suggested. Pisoni *et al.* (1989) suggest a nucleoside carrier system in human fibroblast lysosomes for the transport of these degradation products of nucleic acids.

The susceptibility of ODNs to enzymatic degradation is discussed in section 1.5. The modification of ODN phosphodiester bonds aims to confer resistance to the degradative action of nucleases, as do the conjugation of groups on the 3'-end and 5'-ends. Endonucleases, 3'- and 5'-exonucleases, will not readily recognise these modified bonds. The half-life of ODNs is therefore extended. Phosphatases recognise and cleave terminal phosphates resulting in the liberation of free phosphate groups (Akhtar *et al.* 1991c), which in the case of ^{32}P-ATP 5'-end labelled ODNs would result in cleavage of the radiolabel.

5.1.3 OLIGONUCLEOTIDE BASE COMPOSITION AND LENGTH

ODNs have been modified in various ways in order to increase their efficacy. Two factors, not discussed previously, which may affect ODN association with cells are length and base composition. Optimal lengths and sequence compositions may exist which confer the greatest degree of stability and cellular association.

Base composition has been shown to influence some properties of ODNs. Matsukura *et al.* (1987) found the most active anti-HIV phosphorothioate ODNs have a high GC content, and Stein *et al.* (1988) found the T_m of phosphorothioate ODNs with a high GC content to be higher than those with a high AT content.

Increasing the length of ODNs increases the likelihood of hybridisation to a complementary sequence and increases the T_m of resulting duplexes due to there being a greater number of hydrogen bonds stabilising the structure (Akhtar *et al.* 1991c; Neckers *et al.* 1992). Problems of solubility and folding however become important issues as the length is increased (Akhtar *et al.* 1991c), a compromise between length and T_m must therefore be reached.

Very little information exists on the effects of base composition on cellular association and resistance to degradation, particularly within lysosomes. Iverson *et al.* (1992) report that ODNs rich in purines (adenine and guanine) have a higher cellular uptake compared to pyrimidines (thymine and cytosine), whereas Yakubov *et al.* (1989) observe no such sequence specific effects. Hughes *et al.* (1994) assess homo-ODNs in terms of association to cells and liposomes, G and T bases binding to a greater degree then A and C bases. If such bias exist in terms of cellular association towards one base type then the same may be true of enzymatic degradation rates. ODN length may also affect rate of degradation, *i.e.* an increase in length conferring resistance to degradation.

The effects of these parameters on ODN binding and stability may be a cell line specific phenomenon or a more broad ranging characteristic. Such information would be crucial in the design of an ODN if it were to have a high efficacy.

5.1.4 CIRCUMVENTING LYSOSOMAL DEGRADATION OF OLIGONUCLEOTIDES

The precise effect of conjugating moieties onto ODNs in terms of stability in lysosomes has not been investigated, possibly due to the fact that the mechanism by which various ODNs enter cells has not been clearly defined. Hudson *et al.* (1996a) highlight the need for determining the intra-lysosomal stability of ODNs and development of delivery systems that will circumvent the potential degradation by

lysosomal enzymes. ODNs radiolabelled in different positions were shown to have different $t_{50\%}$ in this study.

Factors such as the nature of the conjugate moiety and ODN chemistry will influence the degradation kinetics of ODNs. The cell line involved will influence ODN stability due to the lysosome content of cells (Steinman *et al.* 1983), for example macrophages are highly endocytic cells and have both internal lysosomal enzyme activity as well as secreting these enzymes (Shepherd *et al.* 1985). Once ODNs have been designed to resist degradation within lysosomes the problem of their transport out of these vesicles can be addressed. Direct insertion of ODNs into the cytosol may in part avoid some degree of degradation, which can be achieved by microinjection (Leonetti *et al.* 1991), although currently this is not a realistic approach.

5.1.5 AIMS - DETERMINING OLIGONUCLEOTIDE STABILITY IN LYSOSOMES

Hudson *et al* (1996a) used a lysosomal preparation composed of rat liver lysosome contents termed 'tritosomes', to assess ODN stability. Our aim was to define the effects of various factors on the rate of degradation in this lysosomal preparation. Factors such as ODN length base composition, backbone chemistry, position of radiolabel and saturation were investigated to define optimal ODN characteristics for resistance to degradation. The stability of the Srev ODN and conjugates (chapter 4) in tritosomes were also investigated. The mono-mannose conjugate (chapter 3) was not assessed due to an insufficient quantity being available.

5.2 MATERIALS AND METHODS

5.2.1 PREPARATION OF TRITOSOMES

Tritosomes were a gift from Dr.R. Duncan (Centre of Polymer Therapeutics, School of Pharmacy, London), prepared from rat liver by a method developed by Trouet *et al.* (1974), see also Hudson *et al.* (1996a) for further details of the preparation of tritosomes. Aliquots of the tritosomes were stored at -20°C until use.

5.2.2 OLIGONUCLEOTIDES

ODNs were synthesised as described in section 2.2.2 and radiolabelled with ^{32}P-ATP in the position indicated (3'-end, 5'-end or internally, section 2.3.1). The homo-ODN sequences used are described in section 4.2.5.1, the backbone chemistry as indicated in each experiment. ODNs antisense to the region complementary to the 3'-splice site of the *tat* gene of HIV-1 in phosphodiester and phosphorothioate backbone chemistries were also synthesised:-

20-mer *tat* antisense sequence

$^{5'}$ GGT AAA AGT CTT AAC CCA CA $^{3'}$

5.2.3 TRITOSOMAL DEGRADATION OF OLIGONUCLEOTIDES

5.2.3.1 Standard Degradation Protocol

Trace quantities of ODNs, approximately 20pica-moles, which were radiolabelled with ^{32}P-ATP (3'-end, 5'-end or internally, section 2.3.1), and used in the degradation experiments. ODN samples were evaporated to dryness with a vacuum dryer in a microfuge tube. A tritosome preparation, 50µl (1mg/ml), was added to the ODN and mixed immediately, following which 5µl samples were removed into 10µl of a stop solution (7M urea, 0.1% w/v bromo-phenol blue and 0.1% w/v xylene cyanol), at the time intervals indicated on the figures. The ODN/tritosome mixture was incubated at 37°C between sampling. Samples were stored at -20°C until electrophoresis. Prior to electrophoresis (7M polyacrylamide urea denaturing gel, section 2.3.2) samples were boiled for 5 minutes and electrophoresed for 1.5 to 2 hours, following which gels were exposed to autoradiography film for 12 hours, at -70°C, in the dark and autoradiographed (section 2.3.2.3). Scanning the autoradiography films and analysing by densitometry (section 2.3.4) performed quantification of ODNs and degradation products.

5.2.3.2 Phosphodiester and Phosphorothioate Backbones

ODNs, 20-mers antisense to the *tat* gene of HIV-I (20tat, section 5.2.2), were synthesised in both backbone chemistries, phosphodiester and phosphorothioate (section 2.2.2), 5'-end radiolabelled with ^{32}P-ATP (section 2.3.1). The standard degradation protocol (section 5.2.3.1) was followed, ODNs samples removed into the stop mixture at the stated time intervals. Samples were electrophoresed alongside a control untreated 5'-end radiolabelled ODN and free untreated ^{32}P-ATP and the gel subsequently subjected to autoradiography and densitometric analysis.

5.2.3.3 The Effect of 3'-, 5'-End and Internal Labelling with ^{32}P-ATP

A phosphorothioate 20tat ODN was synthesised (section 5.2.2) and labelled in different positions, 5'-end or internally (section 2.3.1). A phosphorothioate 7-mer homo-thymidine ODN (7T, section 4.2.5.1) was synthesised and 3'-end labelled. The standard degradation protocol (section 5.2.3.1) was followed for each ODN, samples removed into the stop solution after the indicated incubation periods.

5.2.3.4 The Effect of Oligonucleotide Length

Phosphorothioate homo-thymidines of different lengths, 7-mer, 14-mer, 20-mer and 28-mer (section 4.2.5.1), were synthesised and 5'-end labelled with ^{32}P-ATP and the standard degradation protocol (section 5.2.3.1) followed, samples removed into the stop mixture at the times indicated for each length.

5.2.3.5 The Effect of Oligonucleotide Sequence

Phosphorothioate 7-mer homo-ODNs, 7A, 7G, 7T and 7C (section 4.2.5.1), were synthesised and 5'-end labelled with ^{32}P-ATP and the standard degradation protocol (section 5.2.3.1) followed.

5.2.3.6 The Effect of Increasing Substrate Concentration (Self-Competition)

The phosphorothioate 7-mer homo-thymidine ODN was 5'-end ^{32}P-ATP labelled and assessed for degradation by the tritosomes in the presence of various excess quantities of unlabelled 7T ODN. For saturation studies the ^{32}P-ATP labelled ODN was supplemented with unlabelled 7T ODN for a final concentration of 0.01μM, competed with an equi-molar amount, 100, 1000, 10000, 100000, $1x10^6$ and $2x10^6$ fold excesses

of unlabeled 7T, following the standard degradation protocol (section 5.2.3.1), incubating for 90 seconds before placing samples into the stop solution.

5.2.3.7 The Effect of a Phophatase Inhibitor, Sodium Fluoride

The degradation of phosphorothioate 7T homo-ODN (5'-end ^{32}P-ATP labelled) by tritosomes was assessed in the presence and absence of an inhibitor of the phosphatase enzyme, 10mM sodium fluoride (Sigma-Aldrich Chemicals, Poole, U.K.) (Correll *et al.* 1996), following the standard degradation protocol (section 5.2.3.1).

5.2.3.8 rev Oligonucleotides and Conjugates

The *rev* ODNs, 28Srev, 28Drev, cholhex, C_{16} and HEG, (section 4.1.3, figure 4.1), were 3'-end ^{32}P-ATP labelled and assessed for degradation by tritosomes, following the standard degradation protocol (section 5.2.3.1).

There were insufficient quantities of the mono-mannose conjugate available for assessment of degradation by tritosomes.

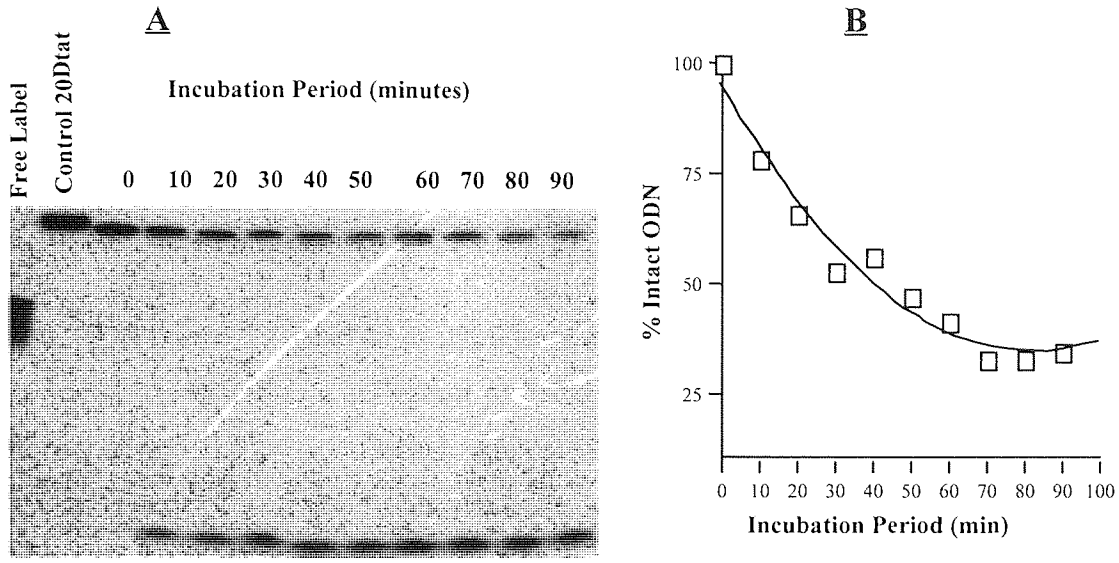
5.3 RESULTS

5.3.1 TRITOSOMAL DEGRADATION OF OLIGONUCLEOTIDES

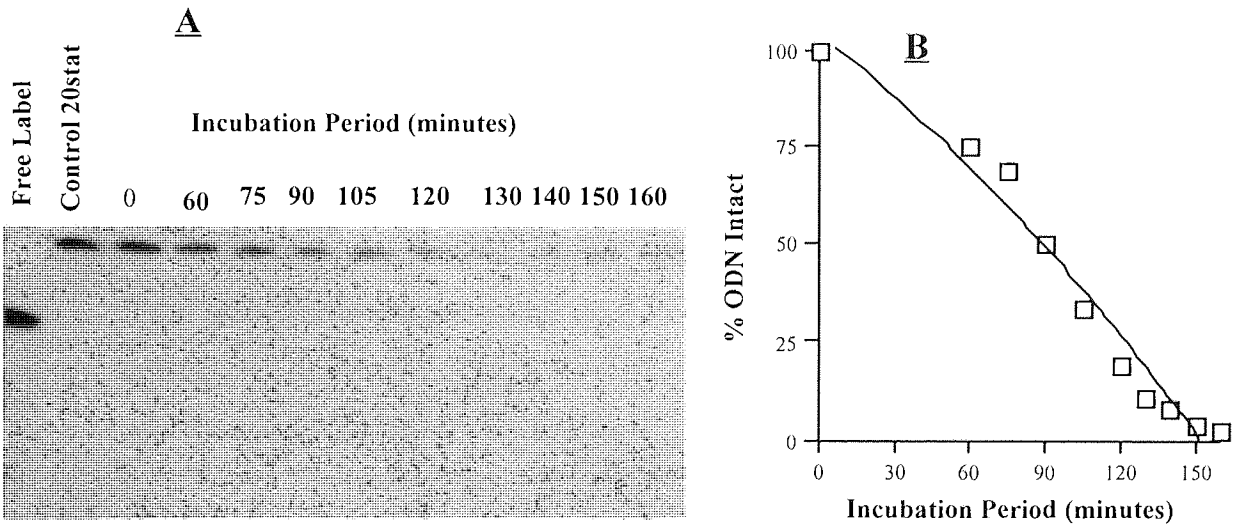
ODNs were evaluated for the time taken for 50% to be degraded by the tritosomes ($td_{50\%}$), as estimated by densitometric analysis of PAGE gel autoradiographs (section 2.3.4). Comparisons were initially made between 5'-end radiolabelled ODNs with phosphodiester and phosphorothioate backbones, ODNs of different length and base composition, degradation at different incubation temperatures and in the presence of excess unlabelled ODN. The effect of an inhibitor of phosphatase enzymes, sodium fluoride, was assessed, following which ODNs radiolabelled at different positions (3'-end, 5'-end or internally labelled with ^{32}P-ATP) were assessed. The degradation of the *rev* ODNs, 3'-end labelled, was also assessed.

5.3.1.1 Phosphodiester and Phosphorothioate Backbone Types

Phosphodiester and phosphorothioate 20tat ODNs, 5'-end radiolabelled, were assessed for degradation by tritosomes as described in section 5.2.3.2 (figures 5.1, 5.2 A and B respectively). The $td_{50\%}$ of the 20Dtat ODN was deduced from densitometric analysis of the scan (figure 5.1A) of electrophoresed samples and the degradation-time profile (figure 5.1B). The 20Dtat ODN degraded to 50% of intact in approximately 40 minutes at 37°C. This correlated with data obtained by Hudson *et al.* (1996a) who found the same ODN, 20Dtat, to have a $td_{50\%}$ of 30 minutes in tritosomes under similar conditions.



Figures 5.1 (A and B): A Representation of the Stability of 5'-End Labelled 20Dtat Oligonucleotide in Tritosomes. Autoradiograph of ^{32}P-ATP-labelled 20Dtat ODN samples incubated with tritosomes at 37°C, 0-90 minutes, and electrophoresed by 20% PAGE (A) and degradation profile, constructed from densitometric analysis, showing the amount of ODN remaining intact at each incubation period (B). Mean of duplicate experiments shown.



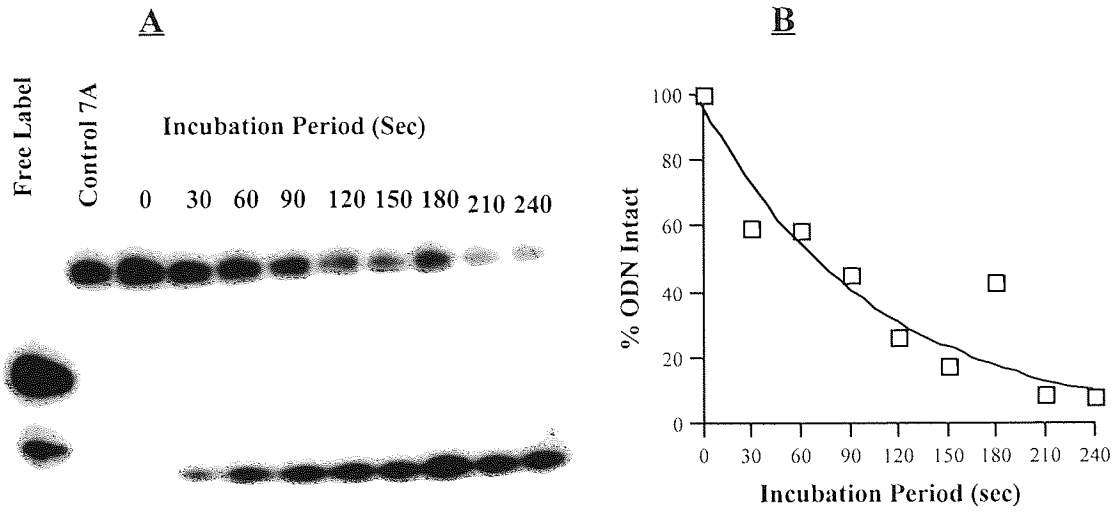
Figures 5.2 (A and B): A Representation of the Stability of 5'-End Labelled 20Stat Oligonucleotide in Tritosomes. Autoradiograph of 5'-end ^{32}P-ATP-labelled 20Stat ODN samples incubated with tritosomes at 37°C, 0-160 minutes, and electrophoresed by 20% PAGE (A) and degradation profile, constructed from densitometric analysis, showing the amount of ODN remaining intact at each incubation period (B). Mean of duplicate experiments is shown.

The 20Stat ODN had a $td_{50\%}$ of 90minutes (figure 5.2) which was the time period found by Hudson *et al.* (1996a) for the same sequence and backbone chemistry (figures 5.2A and B). The PO-ODN, as expected, had a shorter $td_{50\%}$ compared to the corresponding phosphorothioate, which remained intact for approximately a 2-fold longer time period.

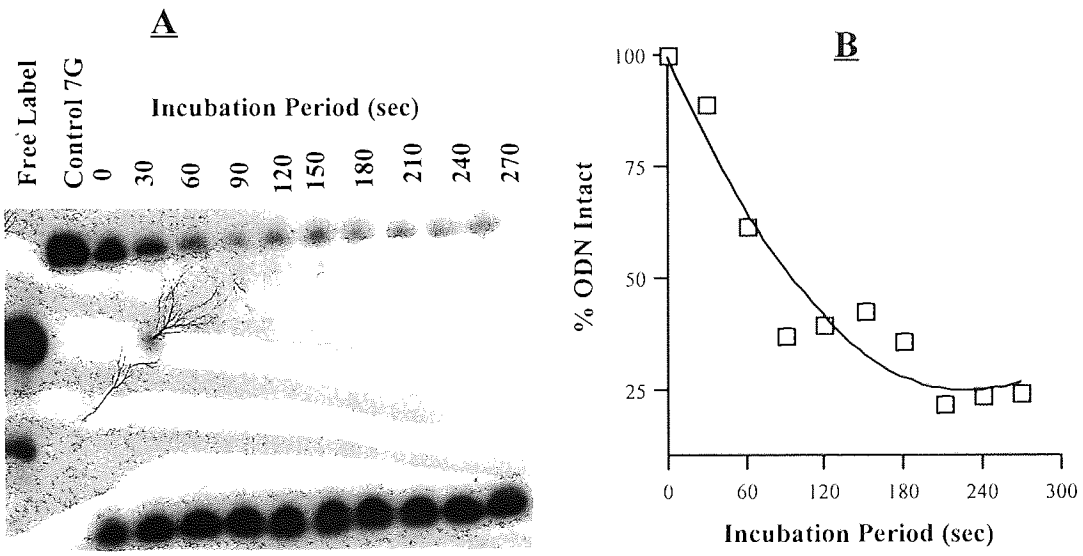
Both ODNs were ^{32}P-labelled at the 5'-end and comprised of the same sequence, the $td_{50\%}$ was therefore a reflection of backbone chemistry only. A degradation profile comprising of a laddering pattern would be expected if digestion of the ODNs were occurring due to enzymatic action, bands of the ladder representing ODNs of different length. Such a pattern was however not seen, raising the question of whether the degradation profile seen by PAGE analysis was true degradation or loss of the end ^{32}Pi (phosphate). The fact that both backbones types have different $td_{50\%}$ does however indicate the influence of ODN chemistry on stability.

5.3.1.2 The Effect of Oligonucleotide Sequence

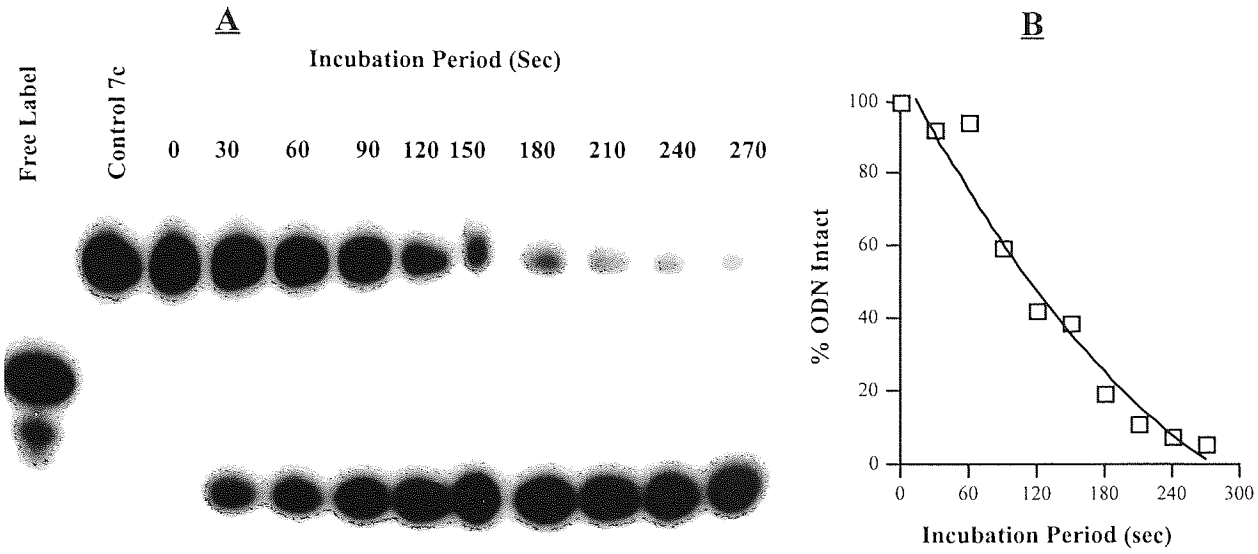
Base composition appears to have a bearing on ODN binding, with 'G' rich sequences having a higher cellular association than the other bases (Hughes *et al.* 1994). Degradation of ODNs consisting of 5'-end radiolabelled homo-T, -A, -G and -C and in the tritosomes was compared, (section 4.2.5.1). Densitometric analysis of the scans and degradation-time profiles provided a value for $td_{50\%}$ for the 5'-end labelled ODNs, 7A, 7G, 7C and 7T (figures 5.3, 5.4, 5.5 and 5.6 respectively), summarised in table 5.21.



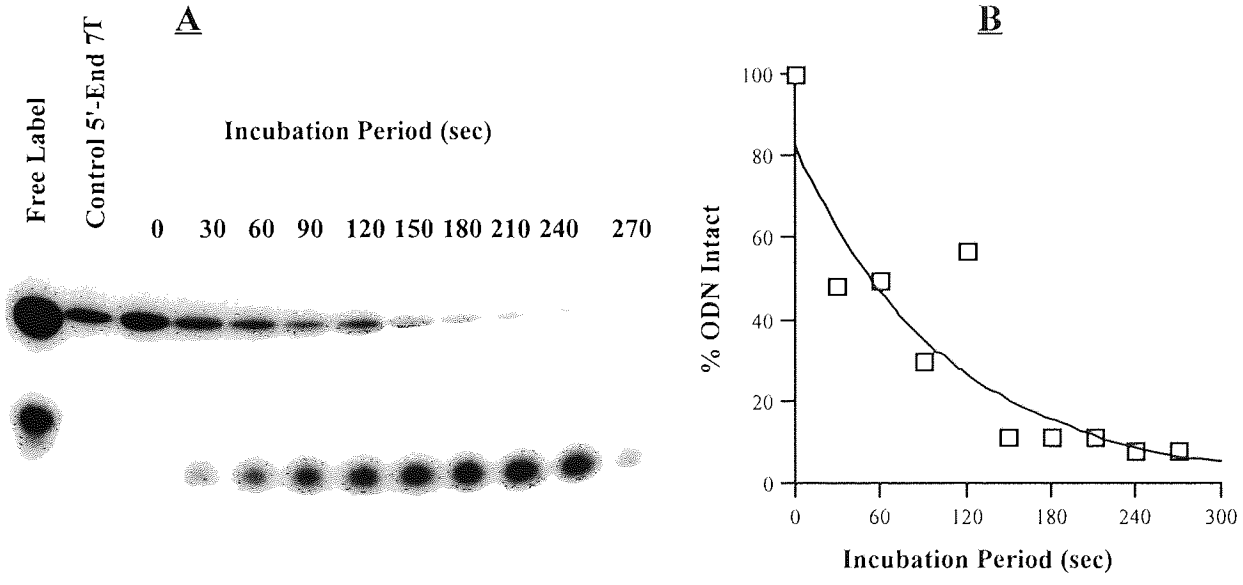
Figures 5.3 (A and B): A Representation of the Stability of 5'-End Labelled 7A Phosphorothioate Oligonucleotide in Tritosomes. Autoradiograph of ^{32}P-ATP-labelled 7A (PS) ODN samples incubated with tritosomes at 37°C, 0-240 seconds, and electrophoresed by 20% PAGE (A) and degradation profile, constructed from densitometric analysis, showing the amount of ODN remaining intact at each incubation period (B). Mean of duplicate experiments is shown.



Figures 5.4 (A and B): A Representation of the Stability of 5'-End Labelled 7G Phosphorothioate Oligonucleotide in Tritosomes. Autoradiograph of 5'-end ^{32}P-ATP-labelled 7G (PS) ODN samples incubated with tritosomes at 37°C, 0-270 seconds, and electrophoresed by 20% PAGE (A) and degradation profile, constructed from densitometric analysis, showing the amount of ODN remaining intact at each incubation period (B). Mean of duplicate experiments is shown.



Figures 5.5 (A and B): A Representation of the Stability of 5'-End Labelled 7C (PS) Oligonucleotide in Tritosomes. Autoradiograph of ^{32}P-ATP-labelled 7C (PS) ODN samples incubated with tritosomes at 37°C, 0-270 seconds, and electrophoresed by 20% PAGE (A) and degradation profile, constructed from densitometric analysis, showing the amount of ODN remaining intact at each incubation period (B). Mean of duplicate experiments is shown.

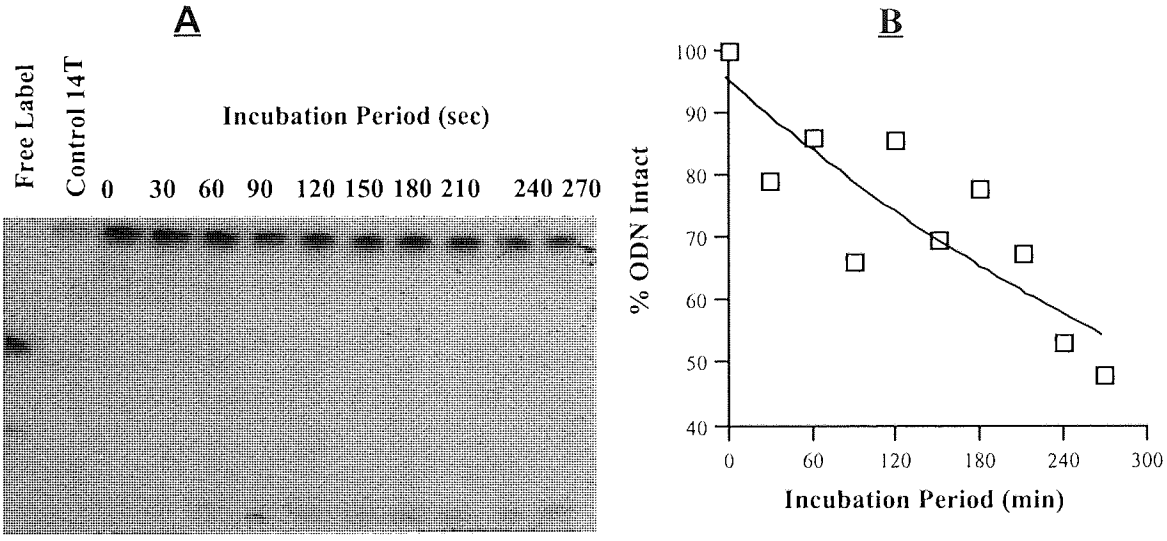


Figures 5.6 (A and B): A Representation of the Stability of 5'-End Labelled 7T Phosphorothioate Oligonucleotide in Tritosomes. Autoradiograph of ^{32}P-ATP-labelled 7T (PS) ODN samples incubated with tritosomes at 37°C, 0-270 seconds, and electrophoresed by 20% PAGE (A) and degradation profile, constructed from densitometric analysis, showing the amount of ODN remaining intact at each incubation period (B). Mean of duplicate experiments is shown.

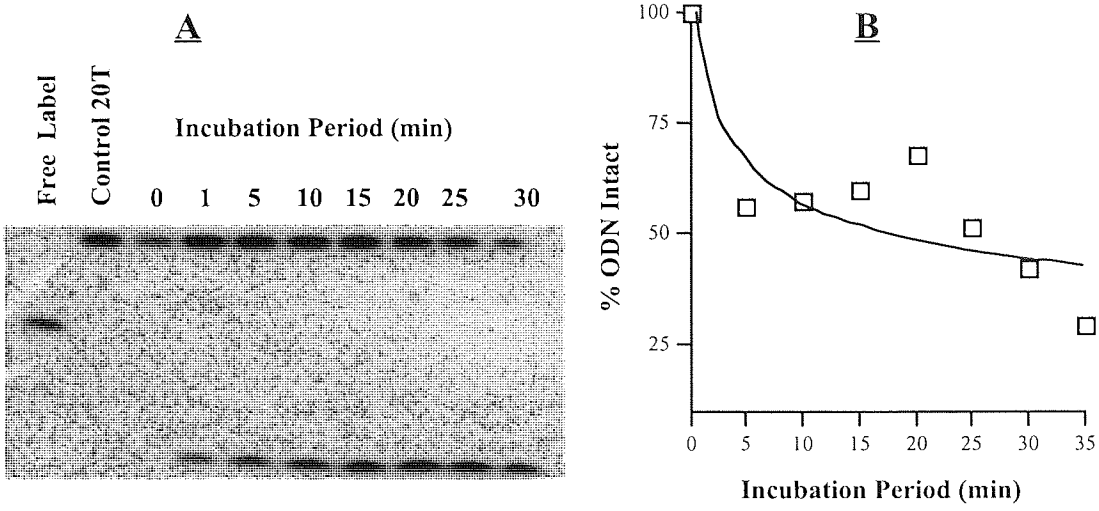
The $td_{50\%}$ of 7A was found to be 75 seconds, 7G to be 90 seconds, 7C to be 110 seconds and 7T to have a $td_{50\%}$ of 60 seconds. Therefore, as with the 20tat ODNs of differing backbone chemistries, base composition also influences tritosomal degradation rates.

5.3.1.3 The Effect of ODN Length

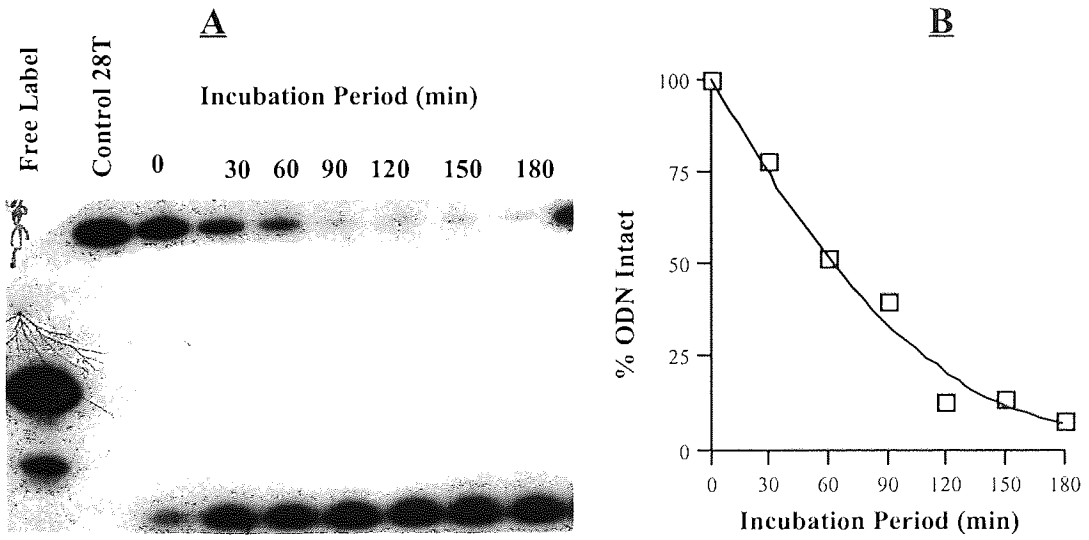
Homo-thymidine ODNs of different length, 7-mer, 14-mer, 20-mer and 28-mer (section 4.2.5.1) were assessed for their $td_{50\%}$ (section 5.2.3.4), and each ODN was labelled at the 5'-end with ^{32}P-ATP. The $td_{50\%}$ for 5'-end radiolabelled 7T (section 5.3.2.2, figure 5.6) was used for comparison to the $td_{50\%}$ of 14T, 20T and 28T. Figures 5.7, 5.8 and 5.9 show scans and degradation profiles for 14T, 20T and 28T ODNs respectively.



Figures 5.7 (A and B): A Representation of the Stability of 5'-End Labelled 14T Phosphorothioate Oligonucleotide in Tritosomes. Autoradiograph of 5'-end ^{32}P-ATP-labelled 14T (PS) ODN samples incubated with tritosomes at 37°C, 0-270 seconds, and electrophoresed by 20% PAGE (A) and degradation profile, constructed from densitometric analysis, showing the amount of ODN remaining intact at each incubation period (B). Mean of duplicate experiments is shown.



Figures 5.8 (A and B): A Representation of the Stability of 5'-End Labelled 20T Phosphorothioate Oligonucleotide in Tritosomes. Autoradiograph of 5'-end ^{32}P-ATP-labelled 20T (PS) ODN samples incubated with tritosomes at 37°C, 0-30 minutes, and electrophoresed by 20% PAGE (A) and degradation profile, constructed from densitometric analysis, showing the amount of ODN remaining intact at each incubation period (B). Mean of duplicate experiments is shown.



Figures 5.9A and B: A Representation of the Stability of 5'-End Labelled 28T Phosphorothioate Oligonucleotide in Tritosomes. Autoradiograph of 5'-end ^{32}P-ATP-labelled 28T (PS) ODN samples incubated with tritosomes at 37°C, 0-210 minutes, and electrophoresed by 20% PAGE (A) and degradation profile, constructed from densitometric analysis, showing the amount of ODN remaining intact at each incubation period (B). Mean of duplicate experiments is shown.

The $td_{50\%}$ of the 7T ODN (figures 5.6A and B) was 60 seconds, 14T ODN was found to have a $td_{50\%}$ of approximately 4 minutes (figures 5.7A and B), an increase in length to 20T resulted in a $td_{50\%}$ of approximately 40 minutes (figures 5.8A and B). The 5'-end 28T ODN had a $td_{50\%}$ of approximately 60 minutes (figures 5..9A and B). The increase in length from 20 to 28 bases, over a 30% increase, produced an increase in $td_{50\%}$ of only 0.33-fold whereas increasing length from 7 bases to 14 produced a 4-fold increase in $td_{50\%}$ and increase from 14 to 20 bases produced a 10-fold increase in $td_{50\%}$.

Theoretically an ODN of longer length would be expected to have a shorter $td_{50\%}$ than an ODN of shorter length, there being a greater surface area, a larger number of potential sites, for enzymatic digestion to occur. However, the opposite result was observed, ODNs of longer length appear to be more stable to degradation by the tritosomes. One possible explanation may be that an increase in stability may result from secondary structure formation by the ODN, which may sterically hinder enzymatic action.

5.3.1.4 The Effect of Increasing Substrate Concentration (Self-Competition)

The effect of increasing the concentration of the substrate, ODN, on the degradation rates of the tritosomes was assessed. The $td_{50\%}$ of a 5'-end labelled 7T ODN incubated with the tritosomes in the presence of excess amounts of unlabelled 7T ODN (method described in section 5.2.3.7) was determined.

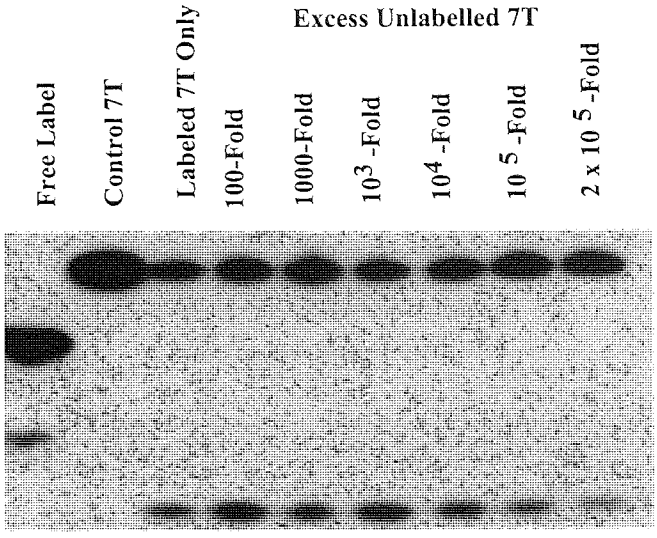


Figure 5.10: A Representation of the Stability of 5'-End Labelled 7T Phosphorothioate Oligonucleotide in Tritosomes in Presence of Excess Unlabelled 7T Phosphorothioate Oligonucleotide. Autoradiograph of 5'-end ^{32}P-ATP-labelled 7T (PS) ODN samples incubated with excess amounts of unlabelled 7T (PS) ODN, with tritosomes at 37°C and electrophoresed by 20% PAGE.

As shown by the scan in figure 5.10, there was a gradual decrease in rate of the degradation of the 7T ODN in the presence of increasing concentrations of unlabelled 7T ODN. In the presence of between 100 and 10^3 fold unlabelled 7T ODN there was no significant change in the amount of degradation observed, compared to the control (labelled ODN only). In the presence of concentrations of 10^3 pmol and above of un-radiolabelled 7T the quantity of intact labelled ODN increased. Approximately 18% and 31% decreases in the amount of degradation was observed in the presence of 10^5 and 2 x 10^5fold unlabelled ODN, respectively. Therefore it is apparent that the enzymes have the capacity to become saturated with substrate/ODN.

5.3.1.5 The Effect of the Phosphatase Inhibitor, Sodium Fluoride

Due to the fact that the tritosomal degradation profiles for all the ODNs thus far did not show a pattern of laddering, the question arose as to whether the ^{32}P-ATP was not simply being lost from the 5'-end of the ODN. If this were the case, the $td_{50\%}$ data may have only indicated phosphatase action (acting at the 5'-end of the

ODN). Sodium fluoride is reported to be a phosphatase inhibitor (Correll *et al.* 1994). To investigate the role of phosphatases in the mixture of enzymes in tritosomes, the $td_{50\%}$ was assessed in the presense and absense of sodium fluoride. Degradation of a 5'-end labelled 7T ODN was monitored (figure 5.11).

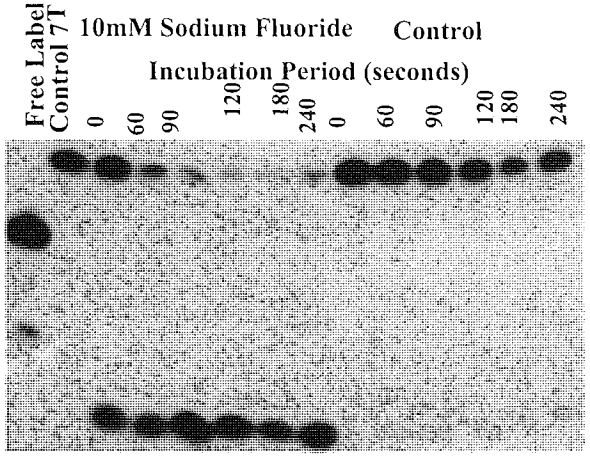


Figure 5.11 : A Representation of the Stability of 5'-End Labelled 7T Phosphorothioate Oligonucleotide in Tritosomes in the Presence of a Phosphatase Inhibitor. Autoradiograph of 5'-end ^{32}P-ATP-labelled 7T (PS) ODN samples incubated in the presence and absence of the phosphatase inhibitor sodium fluoride with tritosomes at 37°C, 0-240 seconds, and electrophoresed by 20% PAGE.

The presence of sodium fluoride virtually abolished degradation of the 7T ODN in the tritosomes as shown in figure 5.11. Incubating the 7T ODN for 240 seconds in the tritosomes, in the presence of sodium fluoride, resulted in 85% degradation compared to no degradation in the absence of this inhibitor. Phosphatase is only one of many types of enzyme found in lysosomes. The specificity of sodium fluoride inhibiting phosphatases in the tritosomes was investigated. It may be the case that other enzymes in the tritosomes are also inhibited. Snake venom phosphodiesterase (SVP), a nuclease enzyme, was investigated for activity in the presence and absense of sodium fluoride (figure 5.12).

Sodium fluoride inhibited the degradative activity of SVP (figure 5.12). Therefore it has been shown that sodium fluoride has the ability to inhibit both phosphatases and SVP, and hence may be an inhibitor of nucleases in general.

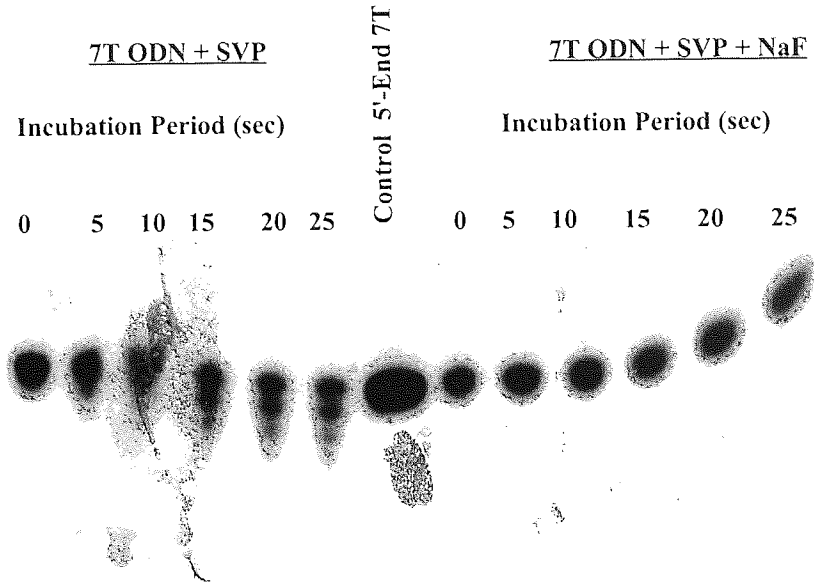


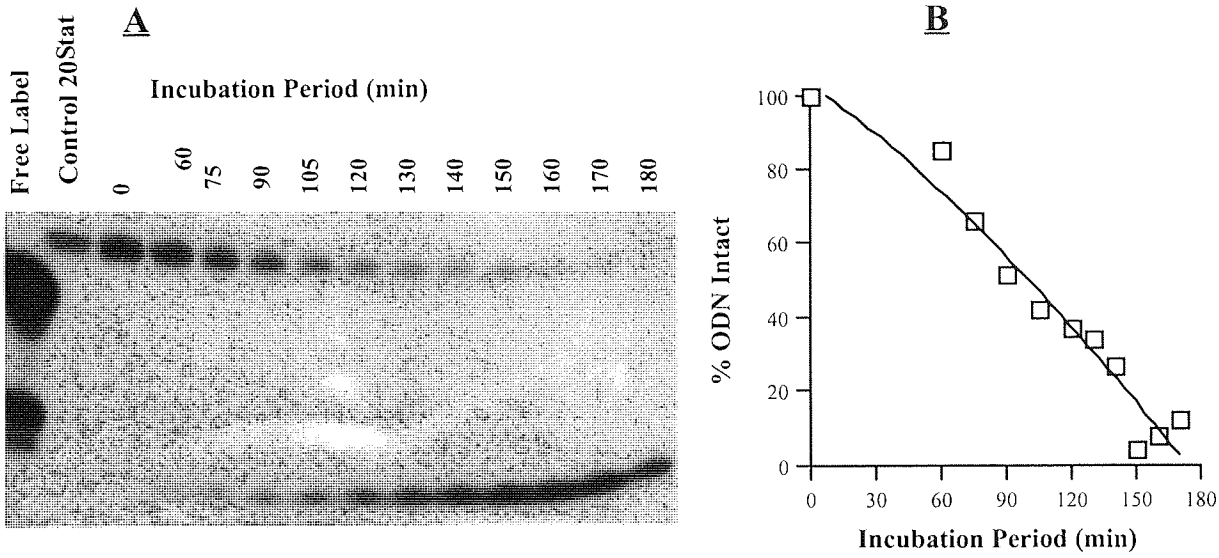
Figure 5.12 : A Representation of the Stability of 5'-End Labelled 7T Phosphorothioate Oligonucleotide in Snake Venom Phosphodiesterase in the Presence of a Phosphatase Inhibitor. Autoradiograph of 5'-end ^{32}P-ATP-labelled 7T (PS) ODN samples incubated in the presence and absence of the phosphatase inhibitor sodium fluoride with SVP at 37°C, 0-25 seconds, and electrophoresed by 20% PAGE.

Previous data suggests that the degradation patterns seen may be as a result of phosphatase activity. Studies using sodium fluoride as an inhibitor did not provide any conclusive evidence for this however due to its inhibitory action on SVP. All subsequent experiments were performed using ODNs radiolabelled at the 3'-end.

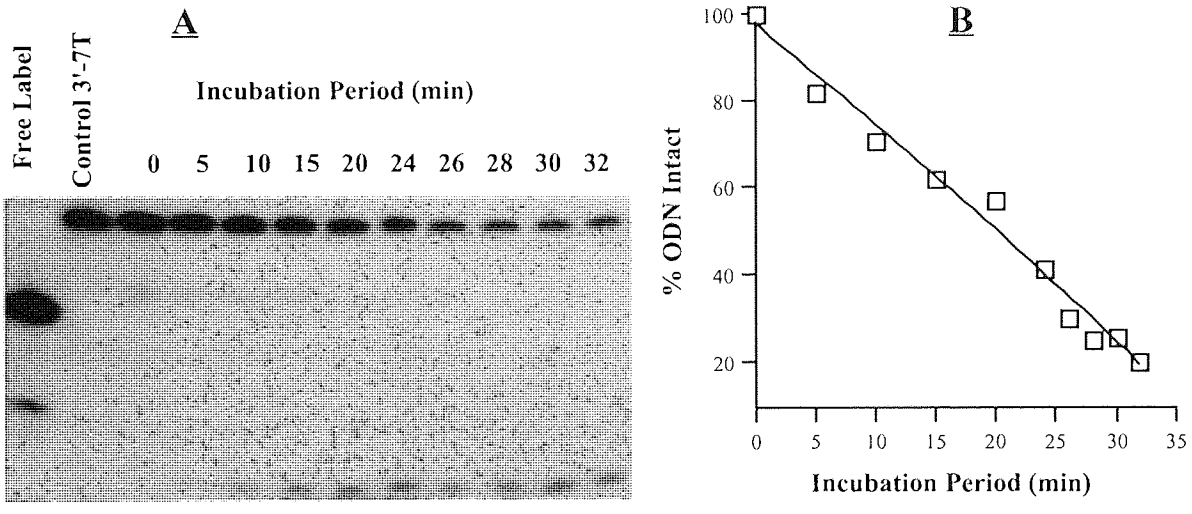
5.3.1.6 The Effect of 3'-End, 5'-End or Internal ^{32}P-ATP Labelling

Degradation profiles for ODNs radiolabelled at different positions were compared. The $td_{50\%}$ of the 5'-end labelled 20Stat (figure 5.2) was compared to that of an internally ^{32}P-labelled 20Stat ODN. Also compared were 5'-end (figure 5.6) and 3'end ^{32}P-labelled 7T phosphorothioate ODNs were also compared as to degradation profiles. The scan and degradation-time profiles for internally ^{32}P-

labelled 20Stat ODN is shown below (figures 5.13A and B), as is the profile for 3'-end 7T ODN (figure 5.14A and B).



Figures 5.13 (A and B): A Representation of the Stability of Internally Labelled 20Stat Oligonucleotide in Tritosomes. Autoradiograph of internally ^{32}P-ATP-labelled 20Stat ODN incubated with tritosomes at 37°C, 0-180 minutes, and electrophoresed by 20% PAGE (A) and degradation profile, constructed from densitometric analysis, showing the amount of ODN remaining intact at each incubation period (B). Mean of duplicate experiments is shown.



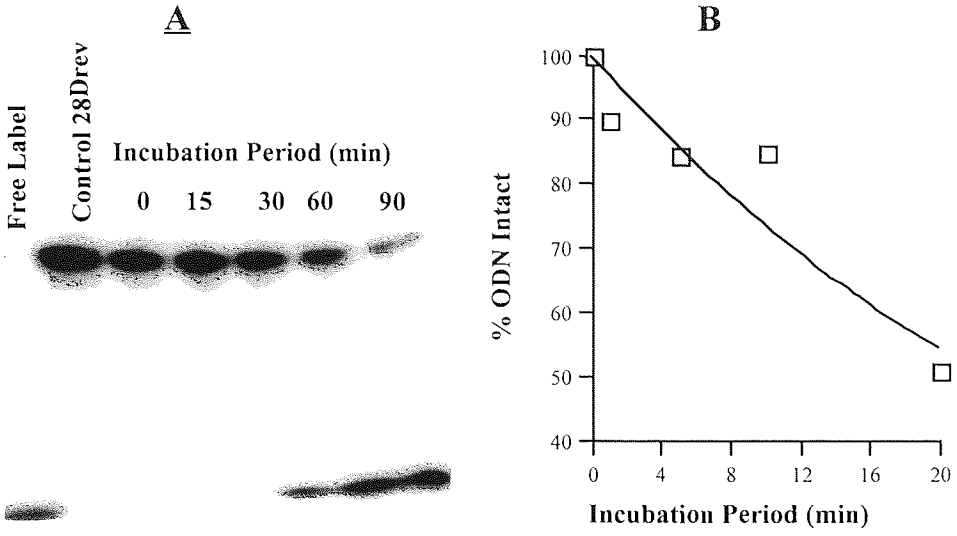
Figures 5.14 (A and B): A Representation of the Stability of 3'-End Labelled 7T Phosphorothioate Oligonucleotide in Tritosomes. Autoradiograph of 3'-end ^{32}P-ATP-labelled 7T (PS) ODN samples incubated with tritosomes at 37°C, 0-32 minutes, and electrophoresed by 20% PAGE (A) and degradation profile, constructed from densitometric analysis, showing the amount of ODN remaining intact at each incubation period (B). Mean of duplicate experiments is shown.

The $td_{50\%}$ of 5'-end and internally labelled 20Stat ODN was found to be the same, 90 minutes (figures 5.2A, B and 5.13A, B respectively). However their profiles differed, the tritosomal degradation profile for the internally labelled 20Stat showed band corresponding to free phosphate, whereas no such band was seen for 5'-end labelled 20Stat. This data not only implicated end phosphate loss from the end of the ODN as shown in figure 5.2A, but also ODN degradation (figure 5.13A), because half the amount of internally labelled ODN was seen after 90 minutes incubation with tritosomes but no free phosphate.

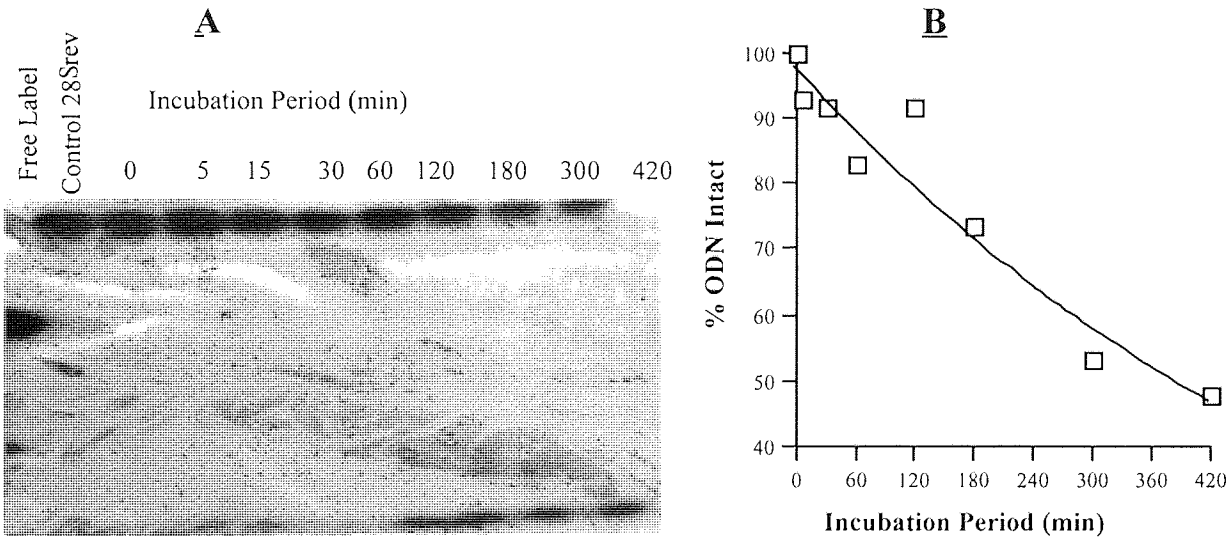
The $td_{50\%}$ of the 3'-end labelled 7T phosphorothioate was found to be approximately 17 minutes (figures 5.14A and B), and the $td_{50\%}$ of 5'-end labelled 7T approximately 1 minute (figures 5.6A and B). A stark difference appears to exist between the $td_{50\%}$ of the 3'-end and 5'-end labelled 7T ODN in the tritosomes at 37°C. As seen by the degradation-time profiles for the 3'-end and 5'-end labelled ODN, there is a difference in the mechanism by which the ODNs were degraded, only 8.5% of the 5'-end labelled ODN remained intact after 4.5 minutes incubation in the tritosomes compared to approximately 70% of the 3'-end labelled 7T ODN. If the same mechanism were involved then a similar $td_{50\%}$ would be expected, both ODNs being identical aside from the orientation of the radiolabel.

5.3.1.7 Srev Oligonucleotide and Conjugate Stability

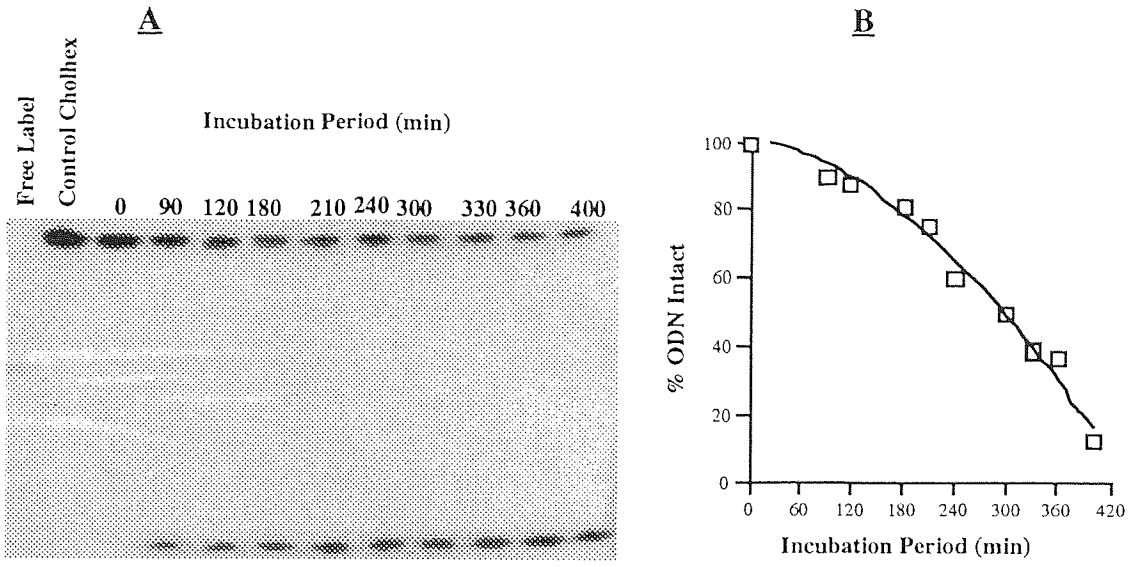
The $td_{50\%}$ in tritosomes of the *rev* ODNs, 28Srev, 28Drev, cholhex, C_{16} and HEG conjugates was evaluated (figures 5.15, 5.16, 5.17, 5.18, 5.19 respectively). The structures and sequences of these ODNs are described in sections 4.2.1 and 4.2.2.



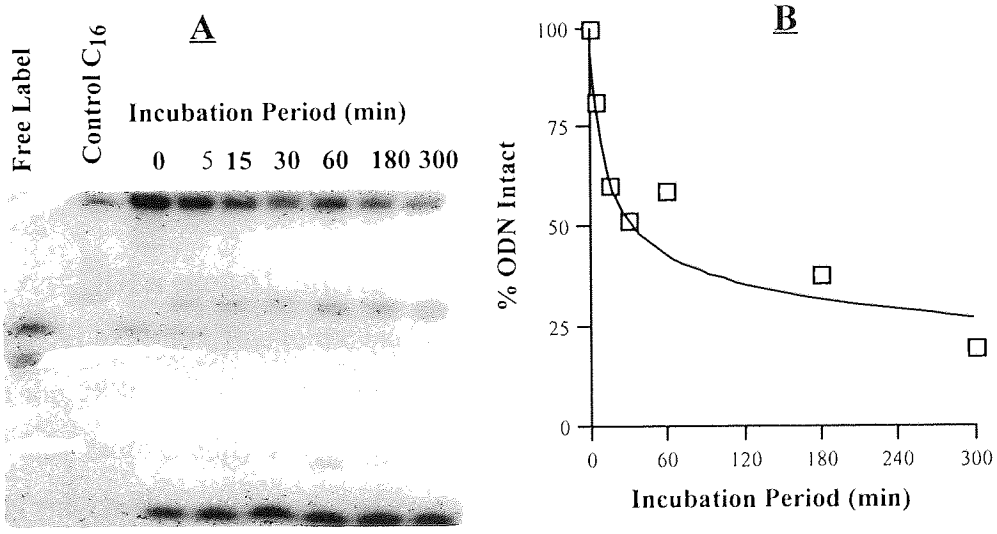
Figures 5.15 (A and B): A Representation of the Stability of 3'-End Labelled 28Drev Oligonucleotide in Tritosomes. Autoradiograph of 3'-end ^{32}P-ATP-labelled 28Drev ODN samples incubated with tritosomes at 37°C, 0-90 minutes, and electrophoresed by 20% PAGE (A) and degradation profile, constructed from densitometric analysis, showing the amount of ODN remaining intact at each incubation period (B). Mean of duplicate experiments is shown.



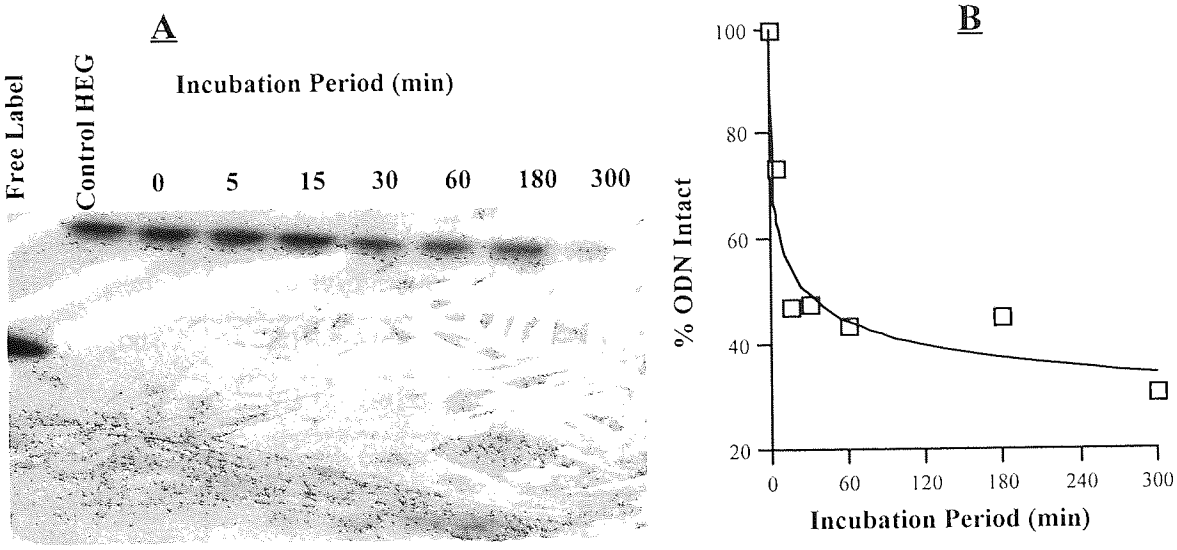
Figures 5.16 (A and B): A Representation of the Stability of 3'-End Labelled 28Srev Oligonucleotide in Tritosomes. Autoradiograph of 3'-end ^{32}P-ATP-labelled 28Srev ODN samples incubated with tritosomes at 37°C, 0-420 minutes, and electrophoresed by 20% PAGE (A) and degradation profile, constructed from densitometric analysis, showing the amount of ODN remaining intact at each incubation period (B). Mean of duplicate experiments is shown.



Figures 5.17 (A and B): A Representation of the Stability of 3'-End Labelled Cholhex Conjugate in Tritosomes. Autoradiograph of 3'-end ^{32}P-ATP-labelled cholhex conjugate ODN samples incubated with tritosomes at 37°C, 0-420 minutes, and electrophoresed by 20% PAGE (A) and degradation profile, constructed from densitometric analysis, showing the amount of ODN remaining intact at each incubation period (B). Mean of duplicate experiments is shown.



Figures 5.18 (A and B): A Representation of the Stability of 3'-End Labelled C_{16} Conjugate in Tritosomes. Autoradiograph of 3'-end ^{32}P-ATP-labelled C_{16} conjugate ODN samples incubated with tritosomes at 37°C, 0-300 minutes, and electrophoresed by 20% PAGE (A) and degradation profile, constructed from densitometric analysis, showing the amount of ODN remaining intact at each incubation period (B). Mean of duplicate experiments is shown.



Figures 5.19 (A and B): A Representation of the Stability of 3'-End Labelled HEG Oligonucleotide in Tritosomes. Autoradiograph of 3'-end ^{32}P-ATP-labelled HEG conjugate ODN samples incubated with tritosomes at 37°C, 0-300 minutes, and electrophoresed by 20% PAGE (A) and degradation profile, constructed from densitometric analysis, showing the amount of ODN remaining intact at each incubation period (B). Mean of duplicate experiments is shown.

The $td_{50\%}$ of each *rev* ODN was evaluated from the scans and degradation profiles (figures 5.15 to 5.219), 28Drev ODN had a $td_{50\%}$ of 40 minutes (figures 5.15A and B), 28Srev and cholhex a $td_{50\%}$ of 300 minutes (figures 5.16 and 5.17 A and B respectively) and both the C_{16} and HEG conjugates a $td_{50\%}$ of 60 minutes (figures 5.18 and 5.19 A and B respectively).

The *rev* ODNs showed different $td_{50\%}$ when incubated with the tritosomes (table 5.20). As expected, due to the backbone chemistry, the 28Drev had a shorter $td_{50\%}$ than the 28Srev, 20 minutes and 300 minutes respectively, the cellular nuclease enzymes more readily recognising and degrading PO-ODNs. Both the 28Srev ODN and cholhex conjugate had a similar $td_{50\%}$ of 300 minutes however. This was an un-expected result, the cholesterol moiety of the cholhex should in part protect the ODN from degradation by 5'-end nucleases thereby prolonging the $td_{50\%}$. The HEG and C_{16} moieties however appeared to increase the degradation rate of the 28Srev ODN. The $td_{50\%}$ of both these conjugates, after 60 minutes, were a fifth of that observed for the unconjugated 28Srev ODN. The tritosomal $td_{50\%}$ of all the ODNs is summarised below in table 5.20.

Table 5.20: Summary of the $td_{50\%}$ of Various Oligonucleotides. Assessed by densitometric analysis of autoradiographs, mean of duplicate experiments.

Position of Radiolabel+ODN	$td_{50\%}$	Backbone Type of ODN	Figure
5'-end-20Dtat	40 +/- 5mins	PO	5.1
5'End-20Stat	90 +/- 8mins	PS	5.2
Internal-20Stat	90 +/- 7mins	PS	5.14
5'End-7A	75 +/- 6secs	PS	5.3
5'End-7G	90 +/- 10secs	PS	5.4
5'End-7C	110 +/- 8secs	PS	5.5
5'End-7T	60 +/- 9secs	PS	5.6
3'End-7T	17 +/- 2mins	PS	5.15
5'End-14T	4.5 +/-1.5mins	PS	5.7
5'End-20T	40 +/- 6mins	PS	5.8
5'End-28T	60 +/- 8mins	PS	5.9
3'End-28Drev	20 +/- 2mins	PO	5.16
3'End-28Srev	300 +/- 14mins	PS	5.17
3'End-Cholhex	300 +/- 19mins	PS	5.18
3'End-C16	60 +/- 11mins	PS	5.19
3'End-HEG	60 +/- 9mins	PS	5.20

5.4 CONCLUSIONS AND DISCUSSION

The localisation of ODNs and ODN-conjugates into endocytic vesicles, endosomes and lysosomes, has been frequently reported following internalisation via an endocytic pathway (Boutorine *et al.* 1993; Noonberg *et al.* 1993; Shoji *et al.* 1996). Such localisation is often characterised as punctuated cytoplasmic fluorescence of ODN-fluorescent labelled conjugates. Lysosomal enzymes degrade nucleic acids to constituent nucleosides (Pisoni *et al.* 1989). If ODNs are to have therapeutic efficacy their release in an intact form into the cytoplasm from these vesicles is essential. The mechanism by which ODNs escape from these vesicles is mainly a matter of conjecture, it is likely that systems will have to be developed that will facilitate release. If RME mechanisms are to be exploited and ODNs internalised into lysosomes the issue of their stability in lysosomal enzymes must first be addressed. The problem of drug encapsulation into lysosomes following endocytosis is addressed by Duncan *et al.* (1980), who investigated the release of carrier molecules from drugs within the lysosomal compartment. Hudson *et al.* (1996a) assessed ODN stability in a lysosomal enzyme preparation and highlighted the necessity for further investigation into circumventing lysosomal degradation. This chapter aimed to evaluate the influence of various factors on stability of oligonucleotides in a rat liver lysosomal preparation.

As expected, phosphorothioates had a longer $td_{50\%}$ than phosphodiesters, 90 minutes and 30 minutes respectively, the data correlating exactly with that of Hudson *et al.* (1996a) who used the same method of investigation and approximately the same concentration of tritosomes. The lysosomal enzymes appear to be inefficient at degrading the PS-ODN, probably due to the inability to recognise the sulphurised backbone compared to the native phosphodiester backbone. Therefore, this data

confirms that PS-ODNs will have a longer $t^1/_2$ in lysosomal enzymes and thus lysosomes.

Investigations into the degradative role played by 3'-end and 5'-end exonucleases in the lysosomal preparation were conducted. Although the radiolabels were conjugated to the ODN as a means of tracking the ODN, they also functioned to confer a degree of protection of the ends of the ODN from enzymatic degradation. A 5'-end ^{32}P-ATP labelled PS-ODN was found to have the same $td_{50\%}$ as an internally labelled counterpart. Comparisons between a 3'-end and 5'-end ^{32}P-ATP labelled 7T PS-ODN revealed a stark difference in $td_{50\%}$, 17 minutes and 1 minute respectively. Aside from the position of the ^{32}P-ATP label there was no difference between the 7T ODNs, the difference in the $td_{50\%}$ therefore due to the action of 3'-end exonuclease enzymes and 5'-end phosphatase enzymes, the former more active in the lysosomal preparation. This data shows that a label on the 5'-end of an ODN confers no increase in stability in the lysosomal preparation, both 5'-end and internally labelled 20Stat ODNs have the same $td_{50\%}$. However, conjugation of a foreign molecule at the 3'-end of an ODN appears to prolong stability in the lysosomal preparation. 3'-end exonucleases appear to be more active than 5'-end phosphatases in lysosomes, as inferred by the greatly increased $td_{50\%}$ for the 3'-end radiolabelled ODN compared to the 5'-end labelled counterpart. Therefore one way to prolong $td_{50\%}$ of ODNs in lysosomes would be 3'-end protection with conjugate moieties.

Assessing the effect of length on $td_{50\%}$ revealed an optimal ODN length for increased stability. Short ODN, 7T and 14T, were degraded rapidly, $td_{50\%}$ of 1 minute and 4 minutes respectively, a 4-fold increase as a result of a 33% increase in length. Increasing the length by a further 30% from 14 to 20 bases again decreased the rate of degradation, a 10-fold increase in $td_{50\%}$ observed (4 and 40 minutes respectively). Further increase in length did not increase $td_{50\%}$ to as great a degree, only a 0.33-fold seen from 20 to 28 bases (40 and 60 minutes respectively). Therefore the increase in stability to degradation appeared to plateau, increasing the length of ODN beyond 20 bases does not appear to confer as significant an increase in $td_{50\%}$.

The 5'-end 14T ODN $td_{50\%}$ of approximately 4 minutes and the subsequent 10-fold increase in stability as a result of increasing the length by 30% makes it apparent that differences in stability were simply not just a function of size alone. If this were the case then a doubling of length is expected to produce a doubling of $td_{50\%}$. This is clearly not the case for this homo-thymidine ODN. An increase in ODN length will increase the chance of non-specific protein binding for phosphorothioates and non-specific binding to target sequences. We can conclude from these studies that ODNs of 20 bases in length will have an optimal $td_{50\%}$ and that any further significant increases in stability will not be achieved by an increase in length.

Each of the four bases differ in their relative hydrophobicities in the following order: C > G > T > A (Hughes *et al.* 1994). This difference influences the ranking order of diffusion across the cellular membrane. The influence of the physiochemical nature of these bases was investigated in terms of susceptibility to enzymatic degradation, as hydrophobicity may render on type of homo-ODN more susceptible to self association and thus less recognisable by enzymes. A difference in $td_{50\%}$ between different nucleotide bases was found. 7C PS-ODN had the longest $td_{50\%}$ of 110 seconds, 7G and 7T sequences both had a $td_{50\%}$ of approximately 90 seconds and the 7A had the shortest $td_{50\%}$ of only 75 seconds. Therefore, resistance to lysosomal degradation appeared to follow the pattern of hydrophobicity. The fact that base composition influences the rate of degradation by lysosomal enzymes will be an important factor in design of ODNs. Those sequences that remain intact within lysosomes for the longest period of time will have the greatest chance of release and therefore reaching their target site.

Saturation of degradative activity was observed in the presence of increasing concentrations of substrate, 7T PS-ODN. The lysosomal enzymes appeared to follow classic saturation enzyme kinetics, whereby the concentration of the enzyme limits the velocity of degradation in the presence of excess substrate. Therefore, achieving a high concentration of ODN within the lysosomes would probably ensure that a relatively high fraction of ODN remained intact long enough for some to exit this organelle into the cytoplasm.

ODN degradation in the lysosomal preparation was confirmed as being due to the action of phosphatase enzymes by using a phosphatase inhibitor, sodium fluoride (Correll *et al.* 1994). 7T PS-ODN degradation was virtually abolished in the presence of this inhibitor, confirming the central role of this enzyme in nucleic acid unstability within lysosomes.

Lysosomal degradation of the *rev* ODNs revealed the 28Srev to have a 15-fold higher $td_{50\%}$ than its phosphodiester counterpart. The higher resistance of the 28Srev ODN to the degradative actions of the lysosomal preparation is higher than can be predicted from backbone chemistry alone, the $td_{50\%}$ for 20Stat being only 3-fold higher than 20Dtat. Such resistance to degradation of the 28Srev may be due to the formation of a secondary structure, which delayed recognition by the lysosomal enzymes (chapter 4.0, section 4.4).

The cholhex conjugate was found to have the same $td_{50\%}$ as the unconjugated 28Srev, 300 minutes, which was suprising. The cholesterol moiety on the 5'-end of the 28Srev ODN should theoretically confer a degree of resistance to degradation from 5'-end exonucleases. Such a protecting effect of the cholesterol moiety not apparent from this data suggests that the degradative enzymes in the lysosomal preparation were able to recognise the 28rev ODN portion of the conjugate regardless of the presence of the 5'-end cholesterol moiety. Both HEG and C_{16} conjugates were found to have shorter $td_{50\%}$ (60 minutes) than the unconjugated 28Srev. A possible explanation for the elevation in degradation rate may be that other enzymes in the lysosomal preparation degrade the HEG and C_{16} groups exposing the 5'-end of the ODN or that any secondary structure the 28Srev ODN formed were hindered sterically by the HEG and C_{16} groups. Increased resistance to lysosomal degradation is the first step in increasing ODN bioavailability. Both the 28Srev ODN and cholhex conjugate appear to be suitable candidates in terms of efficacy, their $td_{50\%}$ of 300 minutes may be sufficiently long enough for release into the cytoplasm.

The lysosomal studies have provided information as to the degradation profiles of various ODNs. Phosphorothioate ODNs, 20-mers in length, protected at the 3'-end

and rich in C bases will have the highest degree of resistance to lysosomal degradation. If ODNs can be designed to remain intact within lysosomes the problems of trafficking them out into the cytoplasm can next be addressed. Although theoretically conjugate moieties may be designed to enhance ODN uptake, their intracellular stability in lysosomes also needs consideration, both HEG and C_{16} moieties had a more rapid degradation rate than the unconjugated ODN. Therefore if endocytosis is to be used of a means of enhancing ODN uptake, lysosomal stability must also be increased.

CHAPTER 6: GENERAL DISCUSSION

A therapy that specifically modulates the cause of a disease state without affecting other entities within the cell would be ideal. ODNs promise such a specific therapy. However, the concept of modulating gene expression is not without problems. Prior to any *in vivo* studies evaluating ODN efficacy, preliminary *in vitro* assessment is essential. Native ODNs, with a phosphodiester backbone, have poor biological stability (Akhtar *et al.* 1992c) and poor cellular uptake (Yakubov *et al.* 1989). The design of more stable ODNs (Loke *et al.* 1989) and development of delivery methods (Letsinger *et al.* 1989; Bonfils *et al.* 1992a; Lappalainen *et al.* 1994) has enabled some ODNs to enter into clinical trials (Azad *et al.* 1995; Zhang *et al.* 1995; Bishop *et al.* 1996; Akhtar *et al.* 1997a).

When an ODN delivery method is evaluated, ODN stability in the cell system under investigation must be assessed as has cellular association and the mechanism of uptake in order to determine intracellular localisation. Delivery methods need to be efficient, non-toxic and specifically deliver ODNs to their target site. Currently an array of methods has been reported to enhance ODN uptake. Some of the methods that have received most attention include physical insertion of ODNs by microinjection (Leonetti *et al.* 1991), encapsulation in liposomes (Lappalainen *et al.* 1997), conjugation to lipophilic moieties such as cholesterol (Letsinger *et al.* 1989) and alkyl groups (Kabanov *et al.* 1990), conjugation of receptor ligands such as mannose (Bonfils *et al.* 1992a and 1992b) or transferrin (Citro *et al.* 1992), conjugation to transferrin receptor antibodies (Walker *et al.* 1995), entrapment into polymer film matrices (Lewis *et al.* 1995) or polymer microspheres (Akhtar *et al.* 1997b).

The intracellular localisation of ODNs is also a major issue with respect to efficacy. ODNs must be delivered to the cytoplasm, the site of mRNA. Many studies have shown putative nucleic acid binding proteins on their surface (Loke *et al.* 1989, Akhtar *et al.* 1996). These binding proteins are hypothesised to bind and internalise their

ligands by RME, therefore resulting in intravesicular (endosomal and lysosomal) localisation. The only process, which ensures localisation in the cytoplasm, is microinjection (Leonetti *et al.* 1991). Therefore, lysosomal stability is an important issue which has not received any significant attention within the ODN research field. If lysosomal localisation cannot be bypassed, intra-lysosomal ODN stability must be increased and a method devised to enable efflux into the cytoplasm. Fusogenic peptides (Bongartz *et al.* 1994, Wyman *et al.* 1997) appear to offer a solution to ODN exit out of lysosomes, studies investigating this approach are currently underway in several different labs.

Both ODN delivery and, excepting the mono-mannose conjugate, lysosomal stability issues were addressed in this project. Two methods to enhance cellular delivery were assessed. A novel mono-mannose ODN conjugate was synthesised, aiming to target the mannose receptor of cultured macrophages and three ODNs, conjugated to different lipophilic groups, cholesterol (cholhex), hexa-ethylene glycol (HEG) and 16-carbon alkyl (C_{16}) moiety, were assessed for enhancement of ODN cellular association. The stability of various ODNs in a rat lysosomal extract was evaluated in order to ascertain what parameters govern stability.

A novel mono-mannose phosphoramidite compound that could be conjugated directly onto an ODN on a DNA synthesiser was designed and synthesised within the department (Akhtar *et al.* 1995). Mannose conjugated ODNs bind cells surface mannose receptors and internalises by RME as shown by previous studies, however conjugation of mannose to ODNs has involved large linker groups. Bonfils *et al.* (1992a and 1992b) conjugated mannosylated BSA to an ODN and mannosylated strepavidin to biotin-ODN respectively, Laing *et al.* (1996) and Rojanasakul *et al.* (1997) used mannosylated-PLL for conjugation to an ODN and delivery to the mannose receptor.

The present approach eliminated the large protein linker component of the mannose-ODN complex, thereby reducing the conjugate size and its potential for an immunogenic response. Evidence for the conjugation of the mannose

phosphoramidite to a nucleotide on the DNA synthesiser was provided by mass spec analysis, demonstrating feasibility of synthesising a mannose phosphoramidite and use for automated ODN synthesis, and mono-mannose/15D ODN (mannose conjugate) synthesis was further supported by electrophoretic mobility by PAGE. The potential for synthesising other sugar phosphoramidites is now highlighted. As with mannose, conjugation of sugar residues to an ODN have involved linker groups, Wu *et al.* (1992) conjugated the sugar asialoorsomucoid to PLL which was subsequently conjugated to an ODN, targeting the galactose receptor.

These experiments involved synthesis of a mono-mannose conjugate, although the mannose receptor recognises and binds ligands possessing multiple mannose residues, so as to assesses feasibility and stability of synthesis.

Successful mannose phosphoramidite synthesis is described and automated conjugation to an ODN is shown. Two macrophage cell lines preferentially bind mannose conjugated ODN. The mechanism involved appears to be RME, expected if the mannose receptor were binding and internalising the mannose conjugated ODN. To our knowledge, this is the first report of direct mannose conjugation to an ODN and subsequent enhancement of ODN cellular association employing a putative RME mechanism.

The mannose conjugate was stable in RAW264.7 and J774 macrophages and a human glioma cell line U87-MG. The cellular association of a 15D-ODN was enhanced by mannose conjugation in RAW264.7 and J774 cells. RAW264.7 and J774 cells bound the novel mannose conjugate to 30-35% higher degrees than the unconjugated ODN after 1hrs incubation at 4°C compared to 37°C; cellular association reduced by 54% in presence of the metabolic inhibitors NaN_3 and 2-DG; 50% greater degree of protein binding of the mannose conjugate compared to the unconjugated ODN; 60% and 46% reduction in cellular association of mannose conjugate in presence of 250-fold excess mannose conjugate and excess mannose monosaccharide respectively; 56% and 71% cellular association reduction in cellular association in presence of PAO and monensin respectively, which both disrupt the endocytic cycle.

Many studies report efficient cellular delivery of cholesterol conjugated ODNs (Svinarchuk *et al.* 1993; Demirhan *et al.* 1994;), data is sparser for ethylene glycol (Jaschke *et al.* 1994) or alkyl chain (Temsamani *et al.* 1994) conjugated ODNs. The effect of these lipophilic groups, cholesterol (cholhex), hexa-ethylene glycol (HEG) and 16-carbon alkyl (C_{16}), conjugated to a 28Srev ODN in order to enhance cellular association was assessed. All 28Srev ODNs were stable in the presence of RAW264.7 cells and the human epithelial cell line A431 for up to 3hrs incubation at 37°C, in serum free media. Rather than conferring protection from 3'-end nucleases, the C_{16} group appeared to promote ODN degradation in RAW264.7 cells after 5hrs incubation, severe fragmentation of the ODN observed. Two important observations were made from cellular association studies, firstly RAW264.7 cells bound the unconjugated 28Srev ODN to an unusually high degree and secondly HEG and C_{16} conjugation reduced binding regardless of increased ODN lipophilicity.

Unlike other studies which report enhanced ODN binding when conjugated to cholesterol, cellular association of cholhex was only marginally greater than the unconjugated 28Srev ODN, most apparent at incubation periods beyond 3 and 5 hrs. HEG and C_{16} conjugate moieties impeded 28Srev ODN cellular association, between 12-15% for both conjugates after 1hrs incubation compared to 20-25% for 28Srev ODN. The high T-base and G-base content of the 28Srev sequence, 28-mer length and CCGC sequence have been implicated in the high degree of cellular association of the unconjugated ODN. Studies of cellular association of homo-ODNs revealed G-base and T-base ODNs to be higher than A-base and C-base ODNs in RAW264.7 cells, correlating to other published reports (Hughes *et al.* 1994), and the CCGC sequence may be involved in the formation of a secondary structure (Van Dongen *et al.* 1996) which may make the ODN more compact or stable and better able to bind and internalise within cells.

Cellular association profiles for all the 28Srev ODNs differed significantly from that of the fluid-phase marker, mannitol, at 37°C. However, the profiles were similar when the ODNs were incubated at 4°C, indicating an active binding mechanism at 37°C (Nakai *et al.* 1996). This theory was further supported by a 35% reduction in cellular

association in the presence of metabolic inhibitors of all ODNs. Proteolytic enzyme, Pronase, treatment least affected cholhex and HEG conjugates, 13% and 10% of ODN conjugates stripped off RAW264.7 cells respectively whereas 45% of 28Srev ODN and 40% of C_{16} conjugate were removed, the latter two apparently associating with cell surface proteins to a higher degree. 32% of 28Drev ODN was also stripped off by Pronase, implicating a high degree of cell surface protein binding, 28Drev was also affected by a decrease in temperature and presence of metabolic inhibitors, consistent with other studies who have identified putative nucleotide binding proteins in cell membranes (Akhtar *et al.* 1996; Hawley *et al.* 1996).

The rate of cellular ODN exocytosis is a function of intracellular localisation (Tonkinson *et al.* 1994). Besterman *et al.* (1981) suggests that molecules internalised by FPE exist in equilibrium, between endocytosis and exocytosis. Efflux experiments revealed cholhex to exocytose at the slowest rate out of both RAW264.7 and A431 cells, indicating sequestration in deep intracellular compartments (Gao *et al.* 1992), the HEG conjugate also had a slow efflux rate. Both these lipophilic conjugates, compared to the unconjugated 28Srev, internalised into deeper intracellular compartments in which they were retained more efficiently. The C_{16} conjugate had a faster efflux rate compared to the 28Srev ODN, therefore, combined with the fact that C_{16} reduced 28Srev ODN cellular association, indicates that most of this conjugate is either loosely cell surface bound or that any fraction internalised is in shallow peripheral compartments. In RAW264.7 cells, mannitol and 28Drev ODN effluxed out at the fastest rate, consistent with molecules internalised by PFE (Besterman *et al.* 1981), therefore probably sequestered in shallow peripheral compartments.

Limited cell surface binding capacity on RAW264.7 cells was shown by self-competition studies. Conjugate binding was not affected by the presence of excess 28Srev ODN, implying the involvement of the lipophilic groups in binding. Non-nucleic acid polyanions reduced cellular association of all but the cholhex conjugate, ionic interaction of ODNs with surface proteins implicated (Wu Pong *et al.* 1992). Cholhex appeared to bind specifically with a high affinity to RAW264.7 cells. C_{16} cellular association was most reduced by non-nucleic acid polyanions, however if

binding were solely due to ionic interactions with proteins then a decrease in pH would result in an increase in cellular association, however this was not found to be the case. The reason for such conflicting C_{16} cellular association data was not elucidated. A decrease in pH increased cellular association of the ODNs in the order 28Srev > HEG > cholhex > C_{16}. The increase in cellular association of 28Srev and HEG ODNs was found to be a result of increased protein binding, at lower pH, as determined by a Pronase experiment, consistent with other studies (Goodarzi *et al.* 1991; Beck *et al.* 1996).

A reduction in cellular association of the Srev ODNs was observed when cells were treated with monensin, which neutralise acidic compartments (Mollenhauer *et al.* 1990). Efflux of the 28Srev ODN and HEG was also increased by monensin. Therefore, monensin either causes a reduction in cellular association as a result of inhibition of receptor recycling back to the surface or an increase in ODN efflux rate. PAO and chloroquine, which also disrupt the endocytic cycle, were found to reduce cellular association of the 28Srev and HEG ODNs, further implicating RME in binding of these ODNs. Southwestern studies revealed all the 28Srev ODNs were binding three common RAW264.7 cell surface proteins, 29kDa, 45kDa and 55kDa, binding probably largely due to the shared 28Srev ODN portion. Additional proteins binding the 28Srev, cholhex and HEG ODNs were also detected.

Endocytosis of the 28Srev ODN and conjugates, by putative binding proteins, is implicated by these studies. Conjugation of lipophilic moieties does not necessarily enhance cellular association or uptake of ODNs, as found for the HEG and C_{16} conjugates, their poor stability and cellular association making them unsuitable as antisense candidates. Both the 28Srev and cholhex ODNs are efficient in terms of binding and uptake. The unconjugated 28Srev ODN also appears promising as an antisense agent, but may have problems relating to PS chemistry, such as a aptameric effect (binding to a protein and causing an effect) and haematological toxicity. Further intracellular trafficking information is essential in order to determine ODN efficacy.

Although many studies show endosomal and/or lysosomal entrapment of ODNs (Stein *et al.* 1994; Shoji *et al.* 1996), little information exists as to ODN stability in these compartments. ODN length, sequence and presence and position of conjugate moiety were found to influence stability studies in rat lysosomal extracts. Hudson *et al.* (1996a), also from this laboratory, is the only report to evaluate ODN stability in extracts, mimicking intracellular lysosomes. An ODN, radio-labelled with ^{32}P-ATP at the 3'-end, was found to have a td $_{50\%}$ 17-fold greater than a counterpart labelled at the 5'-end when incubated with extracts. Therefore, ODNs appear to be most sensitive to 5'-end exonucleases, consistent with ODN stability reports (Shaw *et al.* 1991). An 20-mer ODN had optimal stability in the lysosomal extract and optimal binding to both RAW264.7 and A431 cells. ODNs of greater length, 28-mer and 36-mer, did not confer any further increases in stability to degradation or binding. An order of poly $(C)_7$ < poly $(G)_7$ < poly $(A)_7$ < poly $(T)_7$ was found for ODN td $_{50\%}$, therefore ODNs rich in C and G bases having greater stability when incubated with lysosomal enzymes.

As expected, the 28Srev ODN had a longer td $_{50\%}$ than the PO counterpart, however cholhex was found to have the same td $_{50\%}$ as the 28Srev, conjugation to the 3'-end not appearing to confer any additional resistance to 3'-end degradation as found with the ^{32}P-ATP label at the 3'-end. The td $_{50\%}$ of HEG and C_{16} was shorter than the 28Srev ODN, again protection of the 3'-end of the ODN was not conferred by these moieties, rather degradation unusually promoted. As cellular association studies show, HEG and C_{16} decreased the cellular association of the 28Srev ODN, possibly due to steric hindrance or the formation of secondary structures. The HEG and C_{16} moieties may have exposed the 28Srev ODN to the lysosomal enzymes, therefore increasing the rate of degradation, consistent with poor stability profiles obtained for HEG and C_{16} in RAW264.7 cells.

These studies show that stability to degradation within lysosomes can be achieved by using ODNs of certain length, base composition and end-protecting groups. If trafficking to lysosomes cannot be avoided following cellular internalisation,

intravesicular stability must be increased, so as to allow mechanisms, which will allow ODNs to escape into the cytoplasm to come into play (Bongartz *et al.* 1994).

Delivering ODNs to cells in culture is very much dependent on cell and ODN type. A method that enhances delivery to one system may not necessarily be the case for another system. Mannose receptor targetting as an efficient ODN delivery method has been proved beyond doubt. However, direct mannose residue conjugation to an ODN is now a real possibility due to mannose phosphoramidite synthesis and successful conjugation to an ODN, this method may be utilised for the potential conjugation of other sugars. The conjugation of lipophilic moieties to ODN to enhance cellular delivery is not straightforward, problems of steric hindrance of the ODN, stability to enzymatic degradation and intracellular trafficking are all affected by such conjugation, as seen from the cholhex, HEG and C_{16} studies. Finally, ODN stability to lysosomal degradation can be enhanced if entrapment cannot be circumvented, ODNs with optimal stability properties can be designed.

The fact that a number of antisense ODNs have entered clinical trials (section 1.11) highlights the therapeutic importance of ODN therapy. Whether these compounds become successful drugs will probably not affect interest in developing an antisense based treatment. Research is constantly investigating the improvement of ODN stability, delivery and intracellular localisation, therefore increasing the likelihood that an ODN fulfilling these requirements will be found. The knowledge that ODNs delivered by receptor mediated mechanisms probably localise in lysosomes means that future delivery efforts will centre around direct ODN delivery to the cytoplasm if mRNA is the target or the nucleus if pre-mRNA or the gene is targeted.

Pharmacokinetic studies have shown ODNs *in vivo* to distribute and be eliminated rapidly from the system (Saijo *et al.* 1994; Temsamani *et al.* 1997). Therefore the future of *in vivo* ODN delivery will probably reside in sustained local release (section 1.9.5) (Whitesell *et al.* 1991; Higgins *et al.* 1993), which will ensure that only target regions will receive the treatment and ODN degradation will be counter-acted by renewed release. Systemic delivery is the method currently employed for ODNs in

clinical trials (table 1.9, section 1.11), however local delivery may circumvent problems of non-specific effects/toxicity, reduced efficacy and may reduce the cost of treatment due to direct dosing.

In conclusion, ODNs will require modifications in order to increase stability and improve delivery. The target disease state will probably dictate the type and mode of ODN delivery system used, and what may be found to have a high efficacy in one system not be the case for another. The conjugation of any moieties to ODNs to improve cellular delivery will have to be observed in conjunction with intracellular localisation.

FUTURE WORK

- Mannose-ODN conjugate binding and intracellular trafficking require further investigation to determine if the mannose receptor is bound and ODN internalisation occurs, possibly by the use of a fluorescent label. Synthesis of di-mannose phosphoramidite and ODN conjugation can be developed in order to increase binding affinity for the mannose receptor.

- The unconjugated 28Srev ODN and cholhex conjugate appear to be very promising antisense agents; stable to degradation and high cellular association. The 28Srev may even prove useful in its unconjugated form. The binding proteins found binding the Srev ODNs and the mechanism by which they work require further investigation. If the unconjugated and conjugated 28Srev bind the same proteins ODNs then the question of whether the lipophilic moieties are necessary will arise.

- Increasing lysosomal stability of ODNs will aid in the development of methods which will allow ODN escape into the cytoplasm, degradation can be delayed until lysosome efflux mechanisms are initiated.

CHAPTER 7: REFERENCES

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