DNA CHIP DESIGNED ANTISENSE OLIGODEOXYNUCLEOTIDES TARGETING EGFR MRNA FOR BRAIN TUMOUR THERAPY

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Summary

Glioblastoma multiforme (GBM) is a malignant brain tumour for which there is currently no effective treatment regime. It is thought to develop due to the overexpression of a number of genes, including the epidermal growth factor receptor (*EGFR*), which is found in over 40% of GBM. Novel forms of treatment such as antisense therapy may allow for the specific inhibition of aberrant genes and thus they are optimistic therapies for future treatment of GBM.

Oligodeoxynucleotides (ODNs) are small pieces of DNA that are often modified to increase their stability to nucleases and can be targeted to the aberrant gene in order to inhibit it and thus prevent its transcription into protein. By specifically binding to mRNA in an antisense manner, they can bring about its degradation by a variety of mechanisms including the activation of RNase H and thus have great potential as therapeutic agents.

One of the main drawbacks to the utilisation of this therapy so far is the lack of techniques that can successfully predict accessible regions on the target mRNA that the ODNs can bind to. DNA chip technology has been utilised here to predict target sequences on the *EGFR* mRNA and these ODNs (AS1 and AS2) have been tested *in vitro* for their stability, uptake into cells and their efficacy on cellular growth, EGFR protein and mRNA. Studies showed that phosphorothioate and 2'O–methyl ODNs were significantly more stable than phosphodiester ODNs both in serum and serum-free conditions and that the mechanism of uptake into A431 cells was temperature dependent and more efficient with the use of optimised lipofectin. Efficacy results show that AS1 and AS2 phosphorothioate antisense ODNs were capable of inhibiting cell proliferation by 69% ±4% and 65% ±4.5% respectively at 500nM in conjunction with a non-toxic dose of lipofectin™ used to enhance cellular delivery. Furthermore, control ODN sequences, 2' O-methyl derivatives and a third ODN sequence, that was found not to be capable of binding efficiently to the *EGFR* mRNA by DNA chip technology, showed no significant effect on cell proliferation. AS1 almost completely inhibited EGFR protein levels within 48 hours with two doses of 500nM AS1 with no effect on other EGFR family member proteins or by control sequences. RNA analysis showed a decrease in mRNA levels of 32.4% ±0.8% but techniques require further optimisation to confirm this.

As there are variations found between human glioblastoma *in situ* and those developed as xenografts, analysis of effect of AS1 and AS2 was performed on primary tumour cell lines derived from glioma patients. ODN treatment showed a specific knockdown of cell growth compared to any of the controls used. Furthermore, combination therapies were tested on A431 cell growth to determine the advantage of combining different antisense approaches and that of conventional drugs. Results varied between the combination treatments but indicated that with optimisation of treatment regimes and delivery techniques that combination therapies utilising antisense therapies would be plausible.

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Abbreviations

2'*O*-Me	2'-*O*-methyl
A,G,C,T,U	adenine, guanine, cytidine, thymidine, uridine
ATP	adenosine triphosphate
BSA	bovine serum albumin
°C	degrees Celsius
cpm	counts *per* minute
CO_2	carbon dioxide
DOPE	dioleoyl phosphatidylethanolamine
DOTMA	N-[1(-2,3-dioleyloxy)propyl]-N,N,N,-trimethylammonium chloride
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DMT	dimethoxytrityl
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECACC	European collection of animal cell cultures
EDTA	ethylenediamine tetra-acetic acid
EGFR	epidermal growth factor receptor
FBS	foetal bovine serum
GBM	glioblastoma multiforme
GDP	guanine diphosphate
GTP	guanine triphosphate
GFAP	glial fibrillary acid protein

HCl	hydrochloric acid
HIV-1	human immunodeficiency virus, type 1
HRP	horseradish peroxidase
kDa	kilo Daltons
LSC	liquid scintillation counting
MAPK	mitogen activated protein kinase
ml,μl	millilitres, microlitres
MP-ODN	methylphosphonate oligonucleotide
mRNA	messenger ribonucleic acid
MWt	molecular weight
OD	optical density
ODN	oligodeoxynucleotide
ON	oligonucleotide
ORN	oligoribonucleotide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PNK	polynucleotide kinase
PO-ODN	phosphodiester oligodeoxynucleotide
PS-ODN	phosphorothioate oligodeoxynucleotides
RNA	ribonucleic acid
RNase-H	ribonuclease H
rpm	revolutions *per* minute
SD	standard deviation

SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TBE	tris-borate EDTA buffer
TEMED	N,N,N',N'-trimethylethylenediamine
TGF	transforming growth factor
TRIS	tris(hydroxymethyl)amino methane
VEGF	vascular endothelial growth factor
v/v	volume *per* volume
WHO	World Health Organisation
w/v	weight *per* volume
w/w	weight *per* weight

Chapter 1.0: Introduction

1.1 Antisense Technology

Development in the pharmaceutical industry is not only centred around the advancement of current drugs on the market but also the development of novel drugs to treat diseases which at present are non-effectively treated, such as brain cancers. Conventional drugs for cancer treatment are non-selective and thus cause many side effects that can be distressing to the patient. Although the current drugs for cancer treatment are effective in some cases, certain cancers can become resistant to drugs and thus do not respond to current treatments. Along with the advancement of new drugs, an understanding into the molecular mechanisms behind diseases is also required so that advances in the field can subsequently only lead to improved treatments.

Antisense based therapeutics is a strategy that has the potential to be a very selective technique that allows the targeting of cancer cells with minimal adverse effects to the normal surrounding cells. Antisense technology takes advantage of the natural tendency of complementary nucleic acids to form in solution through Watson-Crick base pairing. By this attraction, the oligonucleotide can bind to cellular complementary DNA, pre-mRNA or mRNA of a target gene and prevent its subsequent translation. In this way gene expression can be potentially inhibited in a highly specific manner to modulate aberrant gene expression without affecting normal cells.

Since the initial use of antisense technology, the development of the technique has progressed rapidly. Developments have allowed the oligonucleotide to become more stable against nuclease degradation, provided enhanced delivery mechanisms both into the cell and at the target site and have allowed greater specificity to the target molecule through the use of better design strategies.

As advances are made both in molecular biology and in antisense treatment specifically, it is possible that antisense technology will provide the basis for a new generation of therapeutic drugs. Antisense modification of a gene can occur naturally as part of the cells natural regulatory system. However the delivery of synthetic oligonucleotides (ONs) can give a more controlled and targeted system for gene therapy.

1.1.1 Antisense Oligonucleotides (ONs)

Oligonucleotides (ONs) are single stranded chains of nucleotides whose sequences are synthesised to be complementary to specific mRNA sequences within a cell. They can be composed of DNA (oligodeoxynucleotides, ODNs), RNA (oligoribonucleotides, ORNs) or mixtures of the two (chimeric ONs). Antisense oligonucleotides were first utilised by Zamecnik and Stephenson in 1978, to prevent the circularisation of the Rous Sarcoma Virus RNA and thus prevent its viral replication (Zamecnik & Stephenson, 1978). The study proposed the use of antisense ONs for therapeutic purposes by inhibiting the growth of the virus in cells. They targeted the viral RNA with a 13mer ON that was shown to inhibit the infection of the cell with the virus. Since the pioneering work of Zamecnik and Stephenson, a number of ONs have been designed to interact with a number of genes or proteins within a cell and demonstrate inhibition of gene expression.

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It is possible to design specific ODN sequences that will recognise a defective gene within a cell either cellular (e.g. oncogenes, growth factors or receptors) or foreign (e.g. viral), and thus possibly provide a treatment for diseases such as cancer and HIV, especially in cases where the causative genetic factor is known.

1.1.1.1 Advantages and Disadvantages of ONs

One of the main advantages of ONs is their specificity and selectivity compared to more conventional drugs currently on the market. Due to their targeting to specific gene sequences it is possible that they could recognise causative cells from their normal counterparts. In the case of cancer therapy, this would be a distinct advantage over current therapies, as certain tumours do not respond well to chemotherapeutic drugs or radiotherapy. Other tumours can develop drug resistance, however, it is not likely that this would occur with the use of ONs as the cell recognises the molecule as a natural product normally found within the cell (Benner *et al.*, 1997). The specificity of ONs is also attributable to the way in which they bind to the cellular DNA or RNA. This occurs simply through Watson-Crick base pairing, as the ON is designed to be complementary to the cellular sequence, which involves strong hydrogen bonding between particular base pairs. This method is already used by the cell in normal DNA and RNA functions and is thus also a quick and simple mechanism of interaction for the ON.

One of the initial problems that faced the usefulness of ONs as potential drugs was their synthesis. Initially DNA and RNA synthesis was a slow process that was complex and required great skill. An automated method of solid phase synthesis has been developed and has now become widely used in the synthesis of oligonucleotides allowing ODNs to

be synthesised quickly and efficiently (Brown and Brown, 1991). Another disadvantage that faces the use of ONs, is degradation by nucleases within the cell, as they have no protection against these naturally occurring scavengers of RNA and DNA. This fact has also led to the problems that ONs are rapidly cleared by the systemic circulation and thus their time span in the body is limited. The ON also needs to be able to enter the cell and as the molecules are fairly large, hydrophilic molecules they do not easily cross cell membranes. Finally, the ON can only be effective against an aberrant target sequence if that sequence is known. This has provided many problems in the past as the target sequence is not always known and the structure of it can be complex (Milligan *et al.*, 1993). In cases where the mRNA sequence is the target, knowing the linear sequence is not always sufficient as the folding of the mRNA into its secondary structure will have an effect on the interaction of the ON. Many of these problems with antisense have been overcome in recent years but many of them are still hurdles to the success of antisense as a therapeutic tool.

1.1.1.2 Potential Molecular Mechanisms of Action

There are many potential mechanisms of actions by which an ON can interact with and exert its effect on the target, whether it be single stranded DNA or mRNA, some of which have not been investigated in detail and so remain theoretical still. It is evident however that the type of mechanism involved may depend on the mRNA targeted, the target site on the mRNA, the type of cell being targeted and the type of ON employed (Chiang *et al.*, 1991). An overview of possible sites of action of ONs is illustrated in figure 1.1.
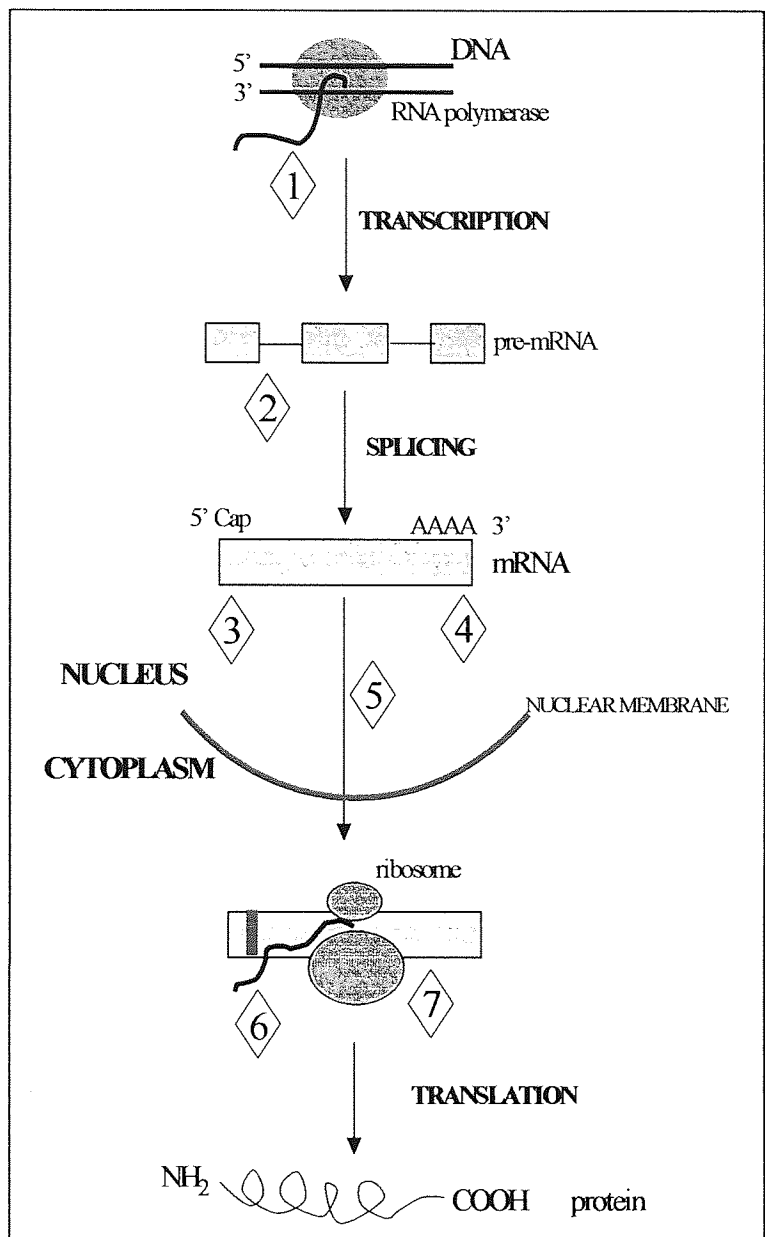
1. Inhibition of transcription either by: triple helix formation or hybridisation to denatured DNA created by RNA polymerase.

2. Inhibition of splicing by splicesomes through hybridisation at intron-exon junctions.

3. Inhibition of translation.

4. Inhibition of polyadenylation

5. Activation of RNase H

6. Inhibition of initiation of translation through inhibition of binding of initiation factors or ribosome assembly at the start codon.

7. Inhibition of ribosome movement.

Figure 1.1: Potential sites of action of antisense oligonucleotides (Adapted from Helene & Toulme, 1990).

The antisense ON can interact with the chromosomal DNA in one of two ways. It can either inhibit the DNA by way of triple helix formation or by preventing the DNA from being transcribed into pre-mRNA. Triple helical DNA can be formed by the binding of the ON in a base specific manner to a stretch of purine bases in the major groove of the DNA. This results in transcriptional arrest of the sense strand of the DNA and thus inhibits protein production (Kashihara *et al.*, 1998). The advantage to this method of inhibition is that it occurs early on in the protein synthesis and thus very few molecules

need to be targeted. However, the homopurine sequences required for triple helix formation limits the number of regions that can be targeted. Furthermore, there are concerns that this method is irreversible and thus may lead to mutagenesis and eventual carcinogenesis. The ON can also bind to partially unwound segments of DNA during transcription thus preventing initiation or elongation of the DNA into its pre-mRNA counterpart.

Binding of ONs to specific mRNA or pre-mRNA sequences may inhibit the interaction of the RNA with proteins, other nucleic acids or other factors required for metabolism of the RNA or its utilisation in the cell. The mechanism by which most ONs are designed to act, is by translational arrest of the mRNA usually by targeting the AUG start codon of the mRNA sequence of interest. This causes steric hindrance preventing molecules such as those that are required to bind to the mRNA from binding. This results in the overall prevention of translation of the mRNA sequence into the protein (Kregnow et al., 1995). This can occur within the cytoplasm, nucleus or both and is the main method of action for ONs such as ORNs or methylphosphonate-ODN (MP-ODNs).

In the nucleus, one potential method of action is the inhibition of RNA splicing, which allows the introns of the pre-mRNA molecule to be removed allowing formation of the mature mRNA species. This process involves splicesomes, which are required to bind to specific sequences on the mRNA and thus binding can be inhibited by the presence of the ON. Binding of the ODN has also been shown to induce alternative splicing in a cell-free splicing system, thus causing inhibition of the mRNA function (Dominski & Kole, 1993). Furthermore, pre-mRNA can be prevented from maturing into mRNA by the inhibition of 3' polyadenylation or mRNA can be inhibited from translocating from the nucleus to the

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cytoplasm. Other possible target regions are the 5'- and 3'- end untranslated regions, where binding of the ON may cause conformational changes or destabilisation of the mRNA rendering it useless for processing into the mature protein (Helene & Toulme, 1990).

RNase H is a ubiquitous molecule that degrades the RNA strand of any RNA-DNA duplex and is normally required in the cell for DNA replication. Unlike translational arrest, the RNase H causes degradation of the mRNA, and so it is postulated that this method is an absolute method for inhibiting protein expression. The utilisation of RNase H has been shown to occur *in vitro* and *in vivo* (Dash *et al.*, 1987; Monia *et al.*, 1996), and appears to be the primary pathway for hybrid arrest in many systems that utilise ODN sequences (Walder & Walder, 1988).

1.1.2 Ribozymes

Another approach to the antisense strategy is the use of ribozymes. They are a class of oligoribonucleic acids that have an intrinsic capability of cleaving RNA in a sequence-specific manner, binding to their target by the principle of complementary base formation.

Ribozymes differ from ONs in the respect that they are totally RNA and can act as enzymes in the absence of proteins and other molecules. It was first established that naturally occurring RNA molecules possess the property of self-catalysis in the early 1980's (Cech *et al.*, 1981) and since then the understanding of the function of RNA and the use of ribozymes to target and cleave other RNA species has been exploited.

There are now several classes of ribozymes, differing in structure, size, specificity and mechanism of action that occur naturally both in plants and microorganisms. They all however require divalent metal ions *i.e.* magnesium or manganese to allow stabilisation of the tertiary structure and maximum catalytic activity. Thus they are also called metalloenzymes.

Ribozymes are composed of two domains; a binding domain and an RNA cleaving domain. The binding domain allows the ribozyme to hybridise to the target by Watson-Crick base pairing, whilst the catalytic domain cleaves the target RNA. Following cleavage the mRNA product formed dissociates from the ribozyme, making it available to hybridise to another sequence thus giving large turnover for one molecule.

One of the major advances in ribozyme research was to develop synthetic ribozymes that are trans-acting (*i.e.* intermolecular cleavage between two separate RNA molecules). This allowed for the development of sequence specific trans-acting ribozymes to down-regulate gene expression and to potentially target and cleave a given cellular or viral RNA target species (for reviews see Tanner, 1999; Carola & Eckstein, 1999).

Of all the ribozymes available to date, the hammerhead or hairpin motif is the best characterised and thus the most commonly used. Its small size also allows it to be readily delivered exo- and endogenously and to be easily synthesised *via* automated solid phase chemical synthesis.

Although ribozymes have the potential to be useful antisense therapy molecules their main drawback is their dependence on specific target RNA cleavage sites and their susceptibility to degradation by endonucleases. Although the stability of ribozymes can

be improved in much the same way as those used for ONs (see section 1.2), there are restrictions to which bases can be altered in the sequence enabling catalytic activity to be maintained. Despite some success in the use of ribozymes to target specific genes (Gibson *et al.*, 2000; Giannini *et al.*, 1999) there still remains much work to be done in this field before the use of ribozymes as antisense agents is as common place as that of ONs.

1.1.3 DNA Enzymes

Following the discovery of enzymatic activities of RNA it was shown that deoxy-nucleic acids (DNA) are not solely informational molecules but are also able to catalyse chemical reactions often with efficiency comparable to that of protein enzymes. *In vitro* selection has led to the development of single stranded DNA molecules that can adopt a variety of defined secondary and tertiary structures, some of which are capable of functioning as catalysts in biological conditions. These molecules are known as DNA enzymes (also termed DNAzymes or deoxyribozymes) and are composed of entirely DNA / ODN that can cleave RNA in a sequence specific manner.

DNA enzymes are similar to ribozymes in that they consist of a catalytic domain of about fifteen oligonucleotides and require magnesium ions (Mg^{2+}) for catalytic activity. However, their increased specificity and a higher catalytic activity allow them to surpass even the small size ribozymes (*e.g.* hammerhead) by an order of magnitude. Santaro and Joyce (1997) have developed a DNA enzyme that could be made to cleave almost any RNA target, efficiently and specifically, under physiological conditions (Santoro & Joyce, 1997).

To date no naturally occurring DNA enzymes have been found, however increasing amounts of catalytic DNA molecules, similar in characteristics to ribozymes, are now being produced by *in vitro* selection (for reviews see Sen & Geyer, 1998; Carola & Eckstein, 1999). It is plausible that DNA enzymes are unlikely to occur naturally due to the fact that the DNA within the cell is found within one place and thus the risk of loss of genetic material would be too great. The rapid catalytic activities and stability of DNA enzymes compared to those of ribozymes makes them a very appealing prospect for the future of antisense therapy.

1.2 Modifications of ODNs

In order to overcome some of the problems with the standard ODN structure, namely nuclease degradation, much work has been performed. The modification however needs to be able to perform its task without interfering with the basic interaction of the ODN with its target and thus preventing it from acting. Figure 1.2 below shows some of the types of modifications that are more widely used to date and involves modifications to the phosphodiester internucleotide linkage, bases and to the sugar moieties:
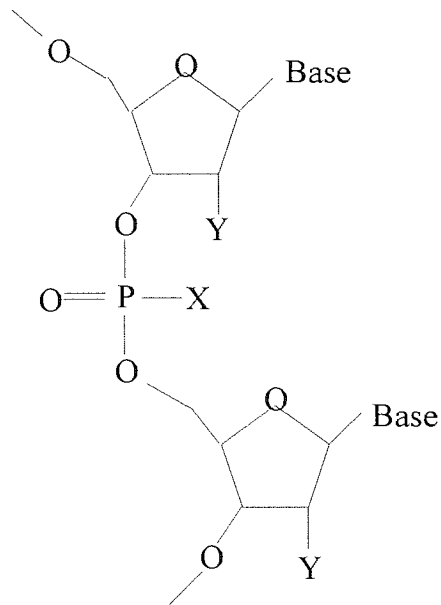
Figure 1.2: A representation of part of a DNA molecule containing the sugar rings, the phosphodiester backbone and the base positions.
X represents modification to the phosphodiester backbone and position Y to the sugar moiety. Modifications are also possible at the individual bases in the sequence.

In a normal unmodified DNA molecule site X represents an oxygen atom and site Y a hydroxyl group (OH). Two of the main modifications found on the phosphodiester backbone (site X) are the addition of a sulphur atom to give phosphorothioate ODNs or the addition of an amine group to give the phosphoramidite ODNs.

1.2.1 Phosphodiester Linkages Modifications

Phosphorothioate ODNs (PS-ODNs) are probably the most widely used modification adapted for antisense work (Marshall & Caruthers, 1993; Eckstein & Gish, 1989) as it has many advantages over the traditional phosphodiester structure. The use of PS-ODNs increased rapidly with the development of fast, effective synthesis techniques that enabled the sulphur to be introduced at an intermolecular linkage of choice (Morvan *et al.*, 1990).

Phosphorothioates allow the phosphodiester backbone to become much more stable to nuclease digestion, both exonuclease and endonuclease, by the addition of a sulphur

group to one of the non-bridging oxygen atoms. This has been shown to be the case in recent studies where the PS-ODNs degrade up to 100 times slower in sera than shown by the equivalent phosphodiester (Akhtar *et al.*, 1991; Wagner *et al.*, 1993). Consequently there has not been any loss of ODN activity associated with phosphorothioate modifications, with the addition of the sulphur atom allowing the recognition of the ODN: mRNA complex by RNase H and thus facilitating the breakdown of the DNA-RNA complex.

The addition of an amine (NH_2) group in the place of one of the non-bridging oxygen's does not seem to hinder the Watson-Crick base pairing of the ODN to the DNA or RNA. They do however form stable triplexes with genomic DNA more readily than other ODNs, in order to prevent gene expression and are highly resistant to exonuclease activity (Shaw *et al.*, 1991). One of the main problems that has been found with this modification is that they are extremely pH sensitive, and thus are easily degraded especially in acidic conditions such as those found in endosomes (Augustine, 1997). Thus delivery of these ODNs is a problem as the typical exogenous uptake mechanisms of ODN into cells involves endosomes (see section 1.3.1).

Methyl group addition at the X position has been shown to give improved stability to the basic ODN structure however uptake studies have shown that it is not saturable thus indicating that the mechanism of uptake for this ODN is via an alternative route to that of receptor-mediated endocytosis. The methylphosphonate ODNs, although extremely stable, do not give RNase H activation thus must act by a different mechanism (Akhtar & Juliano, 1992).

1.2.2 Sugar Moiety Modifications

Sugar modification at the Y position of the diagram shown in figure 1.2 involves esterification. The most commonly found type of this modification is the 2'*O*-methyl modification, which allows the addition of a methyl group at the 2' carbon of the sugar. These analogues have been shown to be among the most stable of all of the modifications employed, but unfortunately do not allow RNase H activation. Thus they do not allow the breakdown of the mRNA by this mechanism but they may still be active by simple steric hindrance of their substrate (Zon, 1988; Inoue *et al.*, 1987). Studies have shown however that chimeric ODNs that combine both the structures of phosphorothioate and 2' *O*-methyl ODNs can be successful as they combine both the 2' *O* -methyl stability and phosphorothioate RNase H active effects that are important factors of ODN stability and activity (Gottikh *et al.*, 1994). These consist of two flanking regions consisting of 2' *O* -methyl modifications, and a central region of phosphorothioate modifications that are capable of binding and initiating RNase H degradation of the target.

1.2.3 Base Modifications

Little work has been carried out using base modifications alone as this is normally accompanied by phosphodiester backbone modifications as well. ODNs containing C-5 propyne pyrimidines (i.e. cytosine, thymidine or uracil bases) have been shown to bind to their target with high affinity and in a specific manner. However, the base modification alone does not allow nuclease resistance and thus this modification is often used in conjunction with phosphorothioate-modified backbones. These combination or mixed

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backbone analogues have been demonstrated to contain nuclease stability, high affinity for their target and possess RNase H activity (Wagner *et al.*, 1993).

1.3 Cellular Uptake and Distribution of Oligodeoxynucleotides

1.3.1 Exogenous Versus Endogenous Delivery

In order to examine the potential inhibitory effects of an ODN on a cellular target, the ODN must be able to enter the cell. One concept of ODN uptake into cells is that of exogenous delivery that permits the uptake of preformed chemically synthesised ODNs into the cell. Endogenous delivery is also a potential method by which ODNs can enter the cell wherein they are delivered to the cell by means of a vector such as a virus, which has been disabled so that it cannot cause harm to the host. Once inside the cell the virus particle can replicate allowing the inserted ODN sequence to be amplified and eventually released into the cellular cytoplasm or nucleus by the same mechanism to which a virus would normally release its viral proteins into an infected cell. Tian *et al* used an endogenously delivered antisense oligonucleotide to *EGFR*, in order to inhibit proliferation and induction of differentiation in human gliomas cells (Tian *et al.*, 1998). This approach was effective in accumulating cells in G0/G1 phase of the cell cycle and of reduction of protein levels and provides a method by which antisense is continuously delivered to the target cell. Although the continuous delivery of the antisense molecule, under the control of a promoter is advantageous, and the fact that the ODN can remain un-modified, thus reducing potential toxicity, scepticism to this approach still remains. Integration of foreign DNA into the genome represents the possibility for alteration of the

genetic structure of the recipient cell. For this reason research is being undertaken into exogenous delivery system for nuclease stable, chemically modified ODNs.

With exogenous delivery, in order for the ODN to enter the cell it must be able to transverse the plasma membrane for which the hydrophilic nature and charge properties of the ODN are not amenable. Despite these hurdles, various cellular uptake mechanisms for ODNs have been described (for review see Akhtar & Juliano, 1992).

ODNs seem to enter the cell by either endocytosis or diffusion, with the faster, endocytic mechanism being the more common. Endocytosis of ODNs can be separated into three distinct types, fluid phase, receptor mediated and adsorptive (Akhtar & Juliano, 1992), with the specific mechanism employed, being dependant upon the type and size of ODN delivered (for review see Reddy, 1996). Loke *et al* (1989) demonstrated that the uptake of ODN into HL60 cells was saturable and dependent upon temperature and ODN length, thus suggesting that the process was specific and an active process (Loke *et al.*, 1989). Indeed an 80kDa receptor was discovered that seemed to bind phosphodiester ODNs and be competed for by PS-ODNs, indicating that a putative receptor protein may be involved in ODN uptake. Research by Yakubov *et al*, (1989) also confirmed this work, using phosphodiester ODNs, however both experimental conditions were debatable as no indication of ODN stability and thus functional activity was examined (Yakubov *et al.*, 1989). It was shown that the methylphosphonate modified ODNs did not compete for the receptor with either PS-ODNs or phosphodiester ODNs and it is thus postulated that these ODNs are delivered in another manner. Conclusions regarding PS-ODN uptake indicate that the process is concentration dependant, in that concentrations below 1μM uptake is a form of receptor-like mechanism and that above this concentration the mechanism is fluid

phase (Beltinger *et al.*, 1995). The concentration effect does confirm that a receptor is involved as these are shown to be saturable. Methylphosphonates on the other hand were thought to cross the membrane by a more passive mechanism due to the lack of competition with the phosphorothioates. This was however disproved by evidence that showed methylphosphonates are taken up by a fluid phase or adsorptive endocytic mechanism (Shoji *et al.*, 1991).

The accumulation of ODN within discrete particles within the cytoplasm of cells has been shown (Akhtar & Juliano, 1992; Shoji *et al.*, 1991). If the uptake mechanism were endocytic this would explain the containment of the ODNs within endosomes located in the cytoplasm.

The uptake of ODN is cell type dependant (Temsamani *et al.*, 1994) and is still a rather time dependant process that appears inefficient, with the majority of the ODN remaining cell membrane bound. To try and overcome this slow uptake process, various delivery enhancement systems have been investigated.

1.3.2 Enhanced Exogenous Delivery

Due to the poor natural uptake processes for antisense ODNs, various methods have been employed to try and enhance this uptake into cells. Although most of these methods revolved around the complex of DNA with lipid a number of novel methods are now emerging which promise to aid in the hurdle of ODN delivery to its target site.

Liposome encapsulation is a process by which phospholipids are arranged in a bilayer and the nucleotide is encapsulated within its aqueous centre to protect it from nuclease

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attack. Liposomes enter the cell by endocytosis where they can be degraded within the lysosomes and the ODN released into the cytoplasm (for review see Juliano & Akhtar, 1992). Advances in liposomes means that they now have a much longer survival time within the circulation, achieved by modulating the types of lipid used. However, the liposomes are still non-specific in their targeting. Although the use of antibody-conjugated liposomes may overcome this problem and provide a cell-specific effect that will enhance the molecular target specific effect of ODNs, the problem still remains that the encapsulation efficiency of anionic and neutral liposomes is poor and thus the concentration of ODN delivered is low (for recent reviews see Tari, 2000; Hughes *et al.*, 2001).

Cationic lipids, such as lipofectin[TM] have also proved to be useful tools for ODN enhanced cellular uptake. They bind to the ODN in a complex due to their opposing charges and their hydrophobic nature allows them to interact and fuse with the plasma membrane allowing their uptake into endocytic particles (Felgner *et al.*, 1987). Some cationic lipids commercially available such as lipofectin[TM] contain a helper lipid (DOPE) which facilitates the release of the ODN from the lipid complex once in the endosome / lysosome complex and thus present it in a bioavailable form to the cell (Roh *et al.*, 2000). Cationic lipids have been used in the delivery of PS-ODNs against *ICAM*-1 mRNA and shows enhanced cytoplasmic and nuclear localisation of the ODN within the cell, which leads to increased activity of the ODN due to its greater concentration within the cell (Bennett *et al.*, 1992). Although it has been observed that the ODN is located in the nucleus as well as the cytoplasm when conjugated to lipids, the mechanism of delivery was not understood. The release of the ODN from the lipid and endosome complex has been demonstrated (Marcusson *et al.*, 1998), and the ODN is subsequently trafficked to

the nucleus where it can exert its effect on the mRNA. Recent advances in lipid development have allowed a novel group of fusogenic liposomes that are pH-sensitive, thus at neutral pH they remain complexed with the ODN but as they traverse through the endocytic pathway the pH becomes more acidic and the complex dissociates (reviewed in Hughes *et al.*, 2001). Unfortunately the anionic nature of the complex will probably result in low encapsulation efficiency of the ODN.

Two restrictions are found when dealing with cationic lipids, namely that they can be cytotoxic and that they are rapidly degraded by serum. Thus when using these compounds in cell culture they need to be administered in serum-free media (Felgner *et al.*, 1994) and a toxicity profile must be performed. However, lipid toxicity has been shown to be reduced when conjugated with ODN (Coulson & Akhtar, 1997). When using cationic lipids it has been shown that each ODN / lipid complex has to be optimised for charge ratio and also cell type, ODN length, ODN chemistry, lipid used to name but a few (Islam *et al.*, 2000; Williams & Buzby, 2000). Lipid delivery methods are unlikely to be used *in vivo* however, due to their rapid clearance from the systemic circulation and their relative toxicity.

Another approach to enhanced ODN delivery is coupling to antibodies that will bind to a receptor and thus transport the ODN into the cell by receptor mediated endocytosis. However, this still poses the problem of how to disassociate the ODN from the endosomal complex whilst still maintaining its activity when inside the cell (Walker *et al.*, 1995).

A novel approach to ODN delivery is the use of carrier-peptide oligodeoxynucleotide conjugates. This involves the use of a hydrophobic signal peptide that is recognised by the cell surface and leads to the internalisation of the whole molecule into the cell cytoplasm. Once inside the cytoplasm a hydrophilic nuclear localisation signal allows the ODN to be transferred to the nucleus where it can exert its effect (Chaloin *et al.*, 1998). Morris *et al* (1997) used a chimeric peptide (MPG) consisting of a hydrophobic fusion domain and a hydrophilic nuclear localisation signal derived from SV40 T-antigen (Morris *et al.*, 1997). They showed that the peptide was able to bind ODNs increasing the ODN stability in serum and showing uptake into the cell, using FITC conjugation, within 1 hour. Cellular distribution was nuclear with any free ODN being distributed in cytoplasmic vesicles. Reduction in temperature did not inhibit the process thus indicating that it was a non-endocytic process.

Finally a relatively new delivery approach for antisense ODNs is the use of dendrimers as particulate delivery vehicles. Dendrimers can be synthesised with several functional groups making them a versatile delivery system and due to their defined polymerisation reactions, a reproducible product. The type of dendrimer formed is dependent on the initial monomer used and the building block monomer, giving rise to a range of categories. The most common dendrimer to date is the polyamidoamine (PAMAM) starburst dendrimer consisting of a hydrocarbon core and surface amino groups. It has been found that ODN interact avidly with the dendrimers to form a complex that is sensitive to pH and ionic strength and that not only protects the ODN from degradation but also delivers the ODN to the cell efficiently (Poxon *et al.*, 1996; Yoo *et al.*, 1999)

1.4 Biological Activity

Despite the many problems faced with the use of ODNs, (e.g. design, stability, uptake and mechanisms of action), many reports have been documented that state evidence of antisense specific activity of these molecules. These reports are from a wide range of targets including growth factors and their receptors (Grandis *et al.*, 1998; De Giovanni *et al.*, 1996; Rockwell *et al.*, 1997), signal transduction molecules (Monia *et al.*, 1996), viruses (Zamecnik & Stephenson, 1978; Shoji *et al.*, 1996) and immune cells (Harel-Bellan *et al.*, 1988).

Many of the approaches to antisense therapy have been to target aberrant genes that are usually over-expressed and in doing so cause problems within the cell, generally leading to the formation of tumours. If antisense ODNs can successfully target the mRNA of the aberrant gene and down-regulate it, this will hopefully eliminate the advantage that the cell holds, allowing it to escape normal cellular control. The distinct advantage to this approach is that the ODN can distinguish between the aberrant mRNA and the normal construct and thus is an effective way of specifically targeting cancer cells without other cellular functions being affected.

Zamecnik and Stephenson provided the earliest report of antisense repression on the growth of viruses in 1978 (Zamecnik & Stephenson, 1978), in which an ODN was inserted into the Rous sarcoma virus 35s RNA sequence to stop it from translating and circularising. This led to the realisation that many other RNA sequences might be inhibited in this way to stop protein production.

Inhibition of TGF-α protein expression in head and neck carcinomas has been shown to occur by an antisense mechanism (Grandis et al., 1998), without the use of an enhanced delivery system. The effects of various sequences were recorded with a reduction in cell number ranging from 61% to 94%. A protein level decrease was also found in tumour cells but unfortunately was also found in control cells; thus these results need to be clarified further. The decrease in control cells indicates that the effect seen by the ODN is due to a non-antisense mechanism of action.

The use of ODNs conjugated with liposomes that are conjugated to folate via polyethylene glycol has been reported to act via an antisense mechanism (Wang et al., 1995). The ODN targeted against the EGFR, induced a decrease in cell proliferation (>90%) and a change in cell morphology, thus indicating some mechanistic effect on the growth of the cells. However, no real controls were employed to show that the mechanism of action was solely antisense and thus these results should be taken lightly.

Antisense ODNs targeted against the C-RAF kinase gene has showed that the normal proliferative signals of the C-RAF-1 protein can be inhibited and thus inhibit cell growth (Monia et al., 1996). With the use of a cationic lipid delivery system the number of treated cells was reduced by 80% within 3 days and the mRNA levels reduced by 80% within 13 days of treatment of tumours. This is an important factor in cancer research as C-RAF kinase is responsible for cellular growth and thus stopping this in cancer cells may prevent tumour progression. The same tests were carried out in-vivo and demonstrated highly specific loss of human C-RAF kinase in tumour xenografts and anti-tumour activity that correlated with the loss of mRNA. Moreover, a series of controls using increased mismatches showed decreasing potency in-vitro and the same pattern in-

vivo. The controls used here help to demonstrate the sequence specific antisense mechanism of the ODN, as they show that increasing base changes in the ODN sequence will result in decreased binding to its target and thus a decrease in ODN effect.

The *RAD51* gene, which is expressed in all cells, is responsible for DNA repair mechanisms. In glioma cells, this gene aids in the resistance of these cells to radiotherapy, one of the current treatments employed for this type of tumour. With the use of antisense, this gene has been shown to be suppressed and has thus caused the glioma cells to become more radiosensitive (Ohnishi *et al.*, 1998). This increased sensitivity allows the tumour to be more effectively treated with radiation therapy and has thus been shown to be effective both *in-vivo* and *in-vitro*.

Similarly an antisense ODN targeting *WAF1/CIP1* was delivered using an adenovirus vector system to glioma cells. The Western analysis revealed a significant decrease in WAF1 /CIP1 protein expression levels and the initiation of apoptosis (Ruan *et al.*, 1999). The main advantage gained from this potential therapy was the sensitisation of glioma cells to chemotherapy with cisplatin compared to control sense ODNs. It is hoped here that antisense treatment will render the normally chemoresistant cells more sensitive and thus treatment regimes currently available will be more effective.

Antisense *BCL-2* ODNs have been investigated for their potential use in the treatment of prostate cancer to delay the onset of androgen-independence following castration. *BCL-2* ODN inhibited BCL-2 protein levels in a dose dependant and sequence specific manner when systemically administered to mice 1-day post castration. Furthermore the BCL-2 levels in other tissues were not significantly affected (Miyake *et al.*, 1999).

Much work has now been performed using ODNs *in-vivo* and results have shown that the ODN, when administered both locally and systemically has a desired effect on the target (for review see Crooke, 1998a). Much of the work has been with the PS-ODN derivatives due to their nuclease stability and their fairly well documented mechanisms of action. Although much work has been performed with antisense ODNs that show gene inhibition, there has been great controversy concerning the findings. Much of this has been due to the lack of suitable controls that prove that the mechanism of action of ODNs is by an antisense effect. Originally many studies used an equivalent sense sequence of the ODN as a control that should not bind to the mRNA and thus should not exhibit an effect such as that found with the ODN. However it has often been observed that the sense sequence can bind and exhibit a similar effect to that of the antisense ODN. This mechanism is probably due to DNA triplex formation and interactions with endogenous antisense RNA (for review see Augustine, 1997). It is now widely known that controls such as non-sense ODNs and sequence specific controls such as mismatch and scrambled sequences are more accurate indicators of antisense action. Effects of ODNs, both antisense and controls, on cell proliferation are no longer accepted as suitable for proving a complete antisense mechanism. Instead it is now required to observe protein and mRNA levels in order to evaluate the exact mechanism of action of the treatment (Stein & Krieg, 1994).

1.5 Clinical Trials

In principle, antisense oligonucleotides can be developed and targeted against any gene product and thus are limited only by the extent of genetic information available and a

satisfactory method by which to design the ON. Current clinical trials of antisense oligonucleotides cover a wide range of diseases (see table 1.1) however the greatest impact is expected in such diseases where genetic dysfunction plays a major part in the disease process such as cancer and viral diseases.

ODN	Delivery Route	Target	Therapeutic Field	Trial Phase	Company
Vitravene	Intra vitrial	CMV Retinitis	Anti-viral in HIV patients	On Market	ISIS / CIBA Vision
ISIS 2302	Topical	ICAM-1	Psoriasis	Phase IIa	ISIS
ISIS 2302	Enema	ICAM-1	Ulcerative Colitis	Phase IIa	ISIS
ISIS 3521	Parenteral	PKC-alpha	NSCLC, other cancer	Phase III	ISIS
ISIS 5132	Parenteral	c-RAF kinase	Ovarian and other cancer	Phase IIa	ISIS
ISIS 2503	Parenteral	Ha-RAS	Pancreatic and other cancer	Phase IIa	ISIS
ISIS 14803	Parenteral	HCV	Hepatitis C	Phase II	ISIS / Elan
GEM 231	Intra-venous	PKA-1alpha	Cancer	Phase II	Hybridon/ Methylgene
G3139	Intra-venous	BCL2	Malignant melanoma	Phase III	Genta
MG98	Intra-venous	DNA Methyltransferase	Cancer	Phase I	Hybridon / Methylgene

Table 1.1: Examples of recent ongoing human clinical trials involving antisense oligodeoxynucleotides.
All of the above trials use PS-ODN chemistries with the exception of GEM231 and MG98, which use mixed backbone ODN chemistries.

The only antisense therapy on the market to date is Vitravene (fomivirsen sodium) provided by ISIS / CIBA vision for the treatment of cytomegalovirus retinitis infections in AIDS patients and is administered by repeated injection into the vitreous of the eye (review in Akhtar *et al.*, 2000).

Despite this success other antisense trials have not been so fortunate. One such trial using a PS-ODN targeting *ICAM-1* in Crohn's patients (ISIS 2302) failed to show any efficacy

in patients, however the same ODN is still undergoing trials for other diseases that also over-express ICAM-1 such as psoriasis and ulcerative colitis.

ISIS 5132, an antisense ODN directed to the 3'-untranslated region of the *C-RAF1* mRNA has been shown to inhibit the growth of tumour cells *in vivo* with specific down-regulation of the target mRNA. However, phase II clinical trials of patients with ovarian cancer have shown a reduction in the progression of cancer (review Tamm *et al.*, 2001). Although promising, the specificity of action for this drug needs to be clarified by detailed screening of other mRNA molecules to determine that no other gene expression is being knocked down.

G3139 targeted against the *BCL-2* has been used in clinical trials for patients suffering from malignant melanoma where the knockdown of *BCL-2* by antisense ODN allows increased chemosensitivity (Jansen *et al.*, 1998). This allows the development of therapies in combination with current chemotherapeutic agents to be established which are currently performing well in clinical trials. Tumour cells have been shown to undergo apoptosis due to decreased *BCL-2* levels with the patients suffering little side effects.

PS-ODNs are known to have several non-sequence specific effects and thus trials are now turning to a second generation of oligonucleotide analogues such as those used by hybridon with GEM132 and MG98. These antisense molecules are of mixed backbone oligonucleotide (MBO) chemistry (a combination of two or more ODN chemistries) and it is hoped that although they are currently delivered by intravenous injection, that this chemistry will enable the delivery of future antisense molecules by oral routes.

In summary current clinical trials show some promise for the use of ODN therapy in the clinic however progress has been slow, especially in the development of cancer therapies. In order for the field of ODN therapy to move forward it is necessary for more ODNs that have shown efficacy *in vitro* to be tested in the clinical setting for which they have been developed.

1.6 Design of Effective Oligodeoxynucleotides

To date there is no general rule as to which the best target sequence of the RNA molecule within the cell should be. However, irrespective of the target it must be accessible to the ODN for it to be able to elicit its effect and thus the folding of the RNA is an important factor to consider.

Initially studies were conducted using computer RNA folding programs to predict the structure of the folded RNA and thus identify accessible sites for antisense targeting (Sczakiel *et al.*, 1993). The programs usually predict sites that are free from intramolecular base pairing (such as hairpin loops), however due to the lack of understanding regarding the secondary and tertiary structure of RNA they have proved to be ineffective in predicting suitable target sequences (Milner *et al.*, 1997; Ho *et al.*, 1998; Sohail *et al.*, 1999). A great deal of sites are generally predicted by this type of program which inevitably introduces a great deal of luck as to which sequence to choose that will be effective *in vitro* and *in vivo*. The main problem with computer folding programs is the lack of prediction of secondary and tertiary folding of the RNA and the account of any other proteins that may be bound to the substrate. Furthermore, the mechanisms of heteroduplex formation are poorly understood and thus even with better knowledge of the secondary structure of RNA more insight into the mechanisms of heteroduplex formation

would be required. Many of the antisense ODNs designed using computer folding programs are targeted to the regions surrounding the AUG start codon of the RNA sequence, which seems to be the most accessible area. Studies using these ODN targets have shown a great deal of non-antisense activity of the ODN though, thus indicating that the sites are not as accessible as first thought (Coulson *et al.*, 1996, Storey *et al.*, 1991). It was recognised therefore that alternative methods for identifying accessible regions for ODN binding were required.

A conventional way in which to select an ODN sequence that is antisense effective is to try a series of ODNs to a particular region. This has been successfully employed in some cases where the various combinations are evaluated using cell culture techniques (Monia *et al.*, 1996). However, this technique is extremely time consuming and expensive with active sequences being rare. Monia *et al.*, (1996) found 1/34 sequence specific antisense ODNs in the case of *C-RAF* kinase, whereas Peyman *et al.*, (1995) found only 1 in 100 ODN sequences targeted to the *HSV-1* gene that exhibited reduced cytotoxic effects of the virus. Furthermore, this method does not exhaust all possible binding sites and thus there is a risk that a more effective target site will be missed.

The use of binding affinities for ODN sequences to mRNA targets was evaluated as a method to screen for potential targets of ODN treatment *in vitro* (Stull *et al.*, 1996). They observed that a significant difference in the binding affinity for the target was observed when the RNA target position was shifted slightly. However, to date no biological correlation with these findings has been reported thus potential use of this method for ODN design cannot be evaluated.

In the hunt for a more cost-effective technique to design ODNs to a target site, the method of RNase H mapping has been employed (Ho *et al.*, 1998; Lima *et al.*, 1997). It is already known that RNase H recognises RNA: DNA duplexes and cleaves the mRNA to release the intact DNA and degraded RNA. Combining the targeted RNA with a random library of ODN sequences and RNase H enables a cleavage reaction to occur, in which the RNase H cleaves any combinatorial duplexes of mRNA and ODN. Subsequent analysis of the cleaved fragments enables the mRNA sequence that has been accessible to be established. This technique has been successfully used to determine ODN accessibility sites for the angiotensin type 1 receptor in which the selected ODNs exhibited 50-80% reduction in cell systems and 65% of the protein levels (Ho *et al.*, 1998). There remain problems still with this technique however, in that the sites defined are broad. It has been shown that a shift of one nucleotide along the sequence can have substantial effects upon the efficacy and potency of the ODN upon its target (Monia *et al.*, 1996), thus a more accurate and sensitive technique is required for optimal sequence prediction.

A relatively fast, novel strategy for predicting RNA structure and thus sequence targeting of ODN has been developed recently. DNA chip technology or scanning combinatorial array technology employs scanning combinatorial nucleotide arrays, in which a series of overlapping ODNs of varying length are immobilised upon polymer membrane or glass supports (Southern *et al.*, 1994). Subsequent radiolabelling of the RNA to which the selected ODNs are targeted and 'walking' of the mRNA along the combinatorial array, allows ODNs that hybridise effectively to the mRNA to be identified (for review see Southern, 1996; Sohail & Southern, 2001). This allows each possible combination of ODN sequence, even to a change of one base, to be analysed for specificity and thus enable choices of ODNs that act *via* a non-antisense effect to be reduced. In theory this

technique could be employed to identify all the possible hybridisation sites along a complete RNA molecule. However this could still be very time consuming and so the technique is more often used in combination with RNase H mapping to reduce the length of RNA to be analysed (Milner *et al.*, 1997). Combination of the two techniques would allow initial areas of accessibility by ODN to be established and then allow analysis of these *in vitro* and *in vivo*, in order to define exact sequences of the ODN required. To date there have been no reports published of the evaluation of this technique when applied to mammalian cells. Thus the specificity of ODNs designed by this method and thus the effectiveness of the technology is unknown.

If studies using this technique can produce results that show antisense molecules are effective against their target in a specific manner, then the use of ODNs in clinical research will be greatly increased. It is already widely accepted that the theoretical action of ODNs is an exciting new approach to gene therapy, and steps required to make them more practicably acceptable are further advances in specificity and delivery mechanisms. With the new design approach described here it is hopeful that this will be achievable.

1.7 Glioblastoma Multiforme

Death due to cancer has decreased over the past decade due to an improved understanding of the disease, which has ultimately led to improved diagnosis and treatment. Brain tumours are however still one of the leading causes of death among young children and adults.

Gliomas are the most common primary tumours arising from the brain and account for more than 40% of all CNS neoplasms. It is difficult to distinguish the original cellular

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origin of gliomas due to their heterogeneity both morphologically and biologically. They can be divided into astrocytomas, which account for 75-90% of the gliomas (Bruner, 1994) along with oligodendrogliomas, ependyomas and mixed gliomas. Astrocytomas have been further graded by various systems in the past but perhaps the most consistent classification system to date has been developed by the World Health Organisation (WHO) which grades both astrocytomas and other primary gliomas (Shapiro & Shapiro, 1998).

Grade	Type of glioma	Aggressiveness
1	Pilocytic astrocytic tumours	Low
2	Astrocytomas and Oligodendrogliomas	Medium
3	Anaplastic astrocytomas and anaplastic oligodendrogliomas	High
4	Glioblastomas	Very high

Table 1.2: WHO grading system for gliomas and an indication of the aggressiveness of the tumour types.

Glioblastoma multiforme (GBM) not only constitute a quarter of all astrocytomas but they are the most deadly with the average survival rate being no more than 1 year (Wong et al., 1994). Due to the vast heterogeneity of the tumour it is difficult to pinpoint the cellular origin. However, two major pathways for their occurrence have been proposed, including rapid development without any prior lesion (de novo occurrence) or development from a less aggressive lesion (i.e. anaplastic astrocytomas). Most GBM however are generally considered to derive from astrocytes due to the finding of glial fibrillary acidic protein (GFAP), an astrocytic marker that can be found in the cytoplasm.

Characteristic features of GBM are high mitotic activity and necrosis with vast infiltration of secondary regions of the brain giving the appearance of multifocal gliomas.

The individual cells can either be small with a high nuclear/ cytoplasmic ratio or very large and multinucleated, where the small cells are the most proliferative along with greater aggressiveness. The greater survival of the tumour is also aided to a certain extent by the development of micro vascular proliferation (MVP) giving the tumour its own individual blood supply, along with the ability to avoid recognition by the immune system.

The most recent therapeutic treatments have not significantly improved patient survival, with a patient mortality rate of only 5% or less for the UK population (www.statistics.gov.uk, 1999). Current methods of treatment used in the treatment of GBM are varied but the main initial therapy is surgery. Although surgical procedures are now safe and accurate due to microsurgical techniques, intraoperative ultrasound, lasers and monitoring, the treatment is still not effective in totally eradicating the tumour (Kornblith *et al.*, 1993). Surgery does improve the short-term patient survival but it is impossible to remove the entire tumour especially as certain areas of brain are not feasible to be removed due to their nature and the need for patient quality of life.

Radiotherapy is normally used as a secondary treatment to surgery to try and remove any few remaining tumour cells after surgery. However, GBM exhibit great resistance to radiotherapy and consequently they would require high doses. Large doses are not feasible though due to the sensitivity of the surrounding normal brain matter that would subsequently receive damage from the radiation. Therefore, until more sensitive and specific radiation treatment is developed the use of radiotherapy is limited.

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Brachytherapy is a fairly new technique that uses temporary radioactive implants at 60Gy to treat the tumour. Although the dose is equal to that usually given by external radiation, trials showed that there was a small increase in patient survival but that there was no statistical significance. Radiosurgery is also a development that can be used in conjunction with external beam radiotherapy but again trials have not shown any significant increase in patient survival of GBM especially those of tumour size larger than 4cm.

Chemotherapy is the treatment of cancer with cytotoxic drugs and this avenue has also been used in the treatment of GBM to follow up surgery and radiotherapy. A report in 1993 demonstrated that chemotherapy added to surgery and radiotherapy improved the median patient survival from 9.4 to 12 months (Fine et al., 1993). To date the nitrosurea drugs have proved to be the most effective (Shapiro & Shapiro, 1998) but none so far have increased patient survival by more than a few months.

One of the main problems in administering drugs is the myriad of biological and physiological factors such as the blood brain barrier, heterogeneity and resistance of the cellular population and the unacceptable toxicities of the drugs. Over the past few years, there has been much interest in the use of biodegradable polymer microspheres impregnated with chemotherapeutic drugs (Brem & Lawson, 1999). These polymers allow the drug to be placed topically at the tumour site and avoid systemic toxicity by slow release of the drug over a period of time.

Due to the ability of GBM to avoid an immune response it is feasible to use immunotherapy treatment to attempt to stimulate a stronger immune response. The use of

lymphokine activated killer cells and interleukin-2 has been investigated but its use is limited by the lack of an appropriate delivery system and toxic effects.

As GBM rarely metastasise and are easily monitored they can be easily targeted by genetic approaches and this seems to be the most likely avenue for current research to take. The rapid growth of the tumour cells also makes them ideal targets for gene delivery techniques such as retroviruses as the inserted genes can be synthesised rapidly (Martuza et al., 1991).

In order to target tumours with molecular based technologies it is necessary to determine the underlying genetic problems that have allowed the tumour to develop. Many detailed genetic studies have been performed on malignant gliomas of patients to identify common alterations within the cellular makeup of the tumours. The molecular changes in the tumour depend upon the type of tumour present and in the case of many whether it has developed by a de novo method or from a less, malignant precursor tumour. Figure 1.3 is a schematic diagram of the types of changes that can occur at each stage of tumour development through to the GBM (taken from Sehgal, 1998).

MOLECULAR CHANGES		DIFFERENT STAGES OF GLIOMA

Normal Brain

Loss of chromosome 17p (p53 mutation)
PDGFα Receptor Over expression
Loss of chromosome 22q

Pilocytic Astrocytomas
(WHO grade I)
Low grade astrocytomas
(WHO grade II)

Loss of 13q (RB mutation)
Loss of 9p (INF-α/β, CDKN2)
Loss of 19q
CDK4 gene amplification
MDM2 gene amplification
GLI gene amplification
Cathepsin B and H over expression
uPAR over expression

Anaplastic Astrocytoma
(WHO grade III)

EGFR gene rearrangement/amplification
Loss of chromosome 10 (MMAC1)
Loss of chromosome 18q21 (DCC)
PDGFrα gene amplification
PDGF A- and B- chain over expression
NOS over expression
p21$^{\text{waf-1/cip1}}$ over expression
Loss of Connexion43 expression

Glioblastoma Multiforme
(WHO grade IV)

Figure 1.3: Schematic representation of the molecular changes during the development of human gliomas.
PDGFα - platelet derived growth factor alpha; PDGFr – platelet derived growth factor receptor; RB – retinoblastoma; INF - interferon; CDKN2 – cyclic AMP dependent kinase number 2; *EGFR* – epidermal growth factor receptor; MMAC1 – mutated in multiple advanced cancers 1; DCC – deleted in colon cancer; NOS – nitric oxide synthase; uPAR – urokinase-type plasminogen activator.

Perhaps the most important and certainly the most frequent problems found with GBM are a gain of one or more copies of chromosome 7 (which contains the *EGFR* gene) or loss of chromosome 10. In addition one of the most consistent abnormalities is the presence of double minute chromosomes (DM), which are small pairs of chromosomes lacking a centromere. DM's tend to appear due to a manifestation of over expression of genes and as they are found in over 50% of GBM, they indicate that gene amplification is a key element in the progression of this type of cancer.

The above genetic alterations are all possible targets for molecular approaches in the treatment of GBM. As EGFR has been shown to be amplified in 40% of GBM with a further 33% of these also exhibiting gene rearrangement (Torp *et al.*, 1991) it seemed likely that *EGFR* would be the best target for molecular biological approaches.

1.8 The Epidermal Growth Factor Receptor

1.8.1 Structure of the EGFR

The epidermal growth factor receptor (EGFR) is a single pass transmembrane protein receptor of molecular weight 170kDa (dependent on source of origin) and falls into the category of the subclass 1 receptors. The EGFR has 1186 amino acids residues and is characteristic of its subclass in that it contains three main domains (figure 1.4).

cys

Extracellular

cys
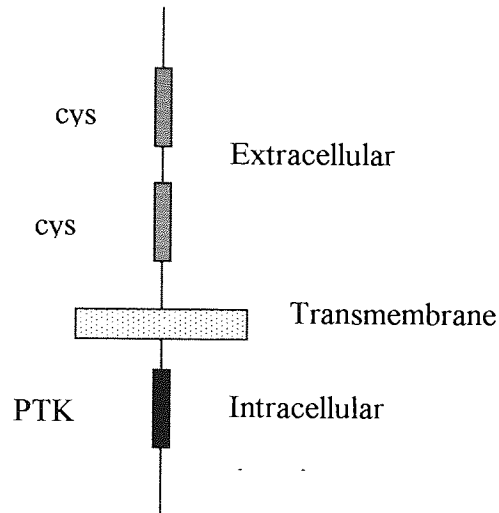
Figure 1.4: A schematic diagram of the EGF receptor.
Cys = cysteine rich domains, PTK = protein tyrosine kinase domain.

Transmembrane

PTK

Intracellular

1.8.1.1 Extracellular Domain

This contains a highly glycosylated ligand binding domain, which for subclass 1 receptors only requires binding of a monomer ligand to activate it. It also contains two cysteine rich domains. With the help of various chimeric experiments, structural characteristics of this domain have been established and helped to clarify the action of each sub-unit in the domain (Lax *et al.*, 1989).

It is proposed that the extracellular domain contains two pairs of sub-units (figure 1.5).
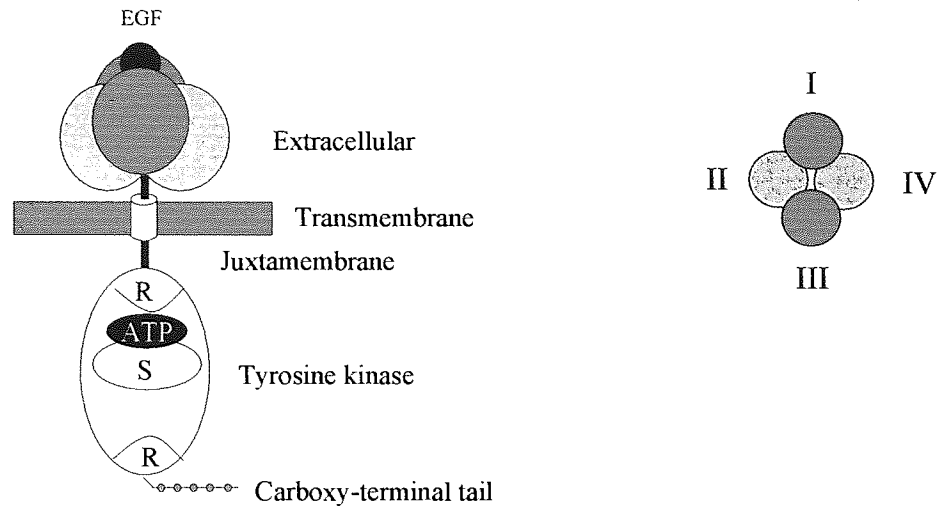
Figure 1.5: Proposed structural diagram of the EGF receptor.
R – proposed tyrosine kinase interaction site for regulatory factors; S – proposed tyrosine kinase interaction site for substrates.

The cleft formed between domains I and III on the above diagram is thought to contribute nearly all of the factors that allow the receptor and ligand to interact with extremely high affinity. Domains II and IV are the cysteine rich domains, which lie close to each other and the plasma membrane, however their precise function is not entirely known. Advances in this area of research have been made with the use of site directed mutagenesis studies; these indicate that the cysteine residues are essential for the biological activity of the receptor (Carpenter & Wahl, 1990).

1.8.1.2 Transmembrane Domain

This region consists of a single hydrophobic sequence of peptides that act as an anchor, to hold the receptor within the plane of the membrane. It simply separates the extracellular and intracellular regions of the receptor and although it is thought that this region has no

significant role in the signal transduction aspect of the receptor, it is possible that it may undergo some conformational change to stabilise the receptor further.

1.8.1.3 Intracellular Region

This region consists mainly of the juxtamembrane domain, the tyrosine kinase domain and the carboxyterminal tail. Of these, the most important is the tyrosine kinase domain and it has been shown that this portion is highly conserved and plays a key role in cell proliferation and differentiation.

Mutations in the cytoplasmic domain have not been widely studied but it has been found that deletions rarely occur in the tyrosine kinase area, but more often in the areas required *for receptor internalisation and inhibitory regions* (Voldborg *et al.*, 1997). These may play a significant role in the constitutive activation of the receptor allowing formation of neoplasms.

The main role of the tyrosine kinase domain is autophosphorylation and thus activation due to ligand binding which ultimately leads to the activation of other intracellular molecules. These active molecules will at length lead to the initiation of gene expression and cell division.

The carboxyterminal tail also possesses autophosphorylation regions that are highly conserved within each receptor subclass. Its role is thought to be that of a negative regulator to the receptor in that autophosphorylation of the tyrosine kinase active site will

give competition to the exogenous molecules thus inhibiting signal transduction (for review see Voldborg *et al.*, 1997).

1.8.2 Signal Transduction of EGFR

The EGFR is activated at the ligand binding domain by numerous ligands, including EGF, transforming growth factor alpha (TGF-α) and amphiregulin (Tang *et al.*, 1997). However, in order to cause the receptor to undergo autophosphorylation the ligand not only has to bind to a receptor, but two receptors are required to dimerise in order for the receptor to become active. The dimerisation allows the transmission of a conformational change to take place between the extracellular and intracellular domains without alteration of the sequence of the transmembrane domain.

Within seconds of the ligand binding, the kinase region becomes activated and can thus phosphorylate the tyrosine residues on the carboxyl terminus. The tyrosine residues are specific and are confined to only three or four of which the targeted residue is dependent on the type of ligand bound and the combination of the two receptors. Once activated these sites become docking areas for a variety of substrates such as exogenous proteins and adapter molecules (Fry *et al.*, 1993). These areas of specificity are now known as SH2 domains (*SRC* homology domain 2) due to their resemblance to the *v-SRC* and *c-SRC* proteins.

The resulting passage of the activated signal towards the nucleus varies depending on the type of ligand bound and the response required and is becoming more and more complex

as molecular biology advances. Figure 1.6 is a diagrammatic representation of the major

pathway that may occur upon ligand binding to the EGFR.
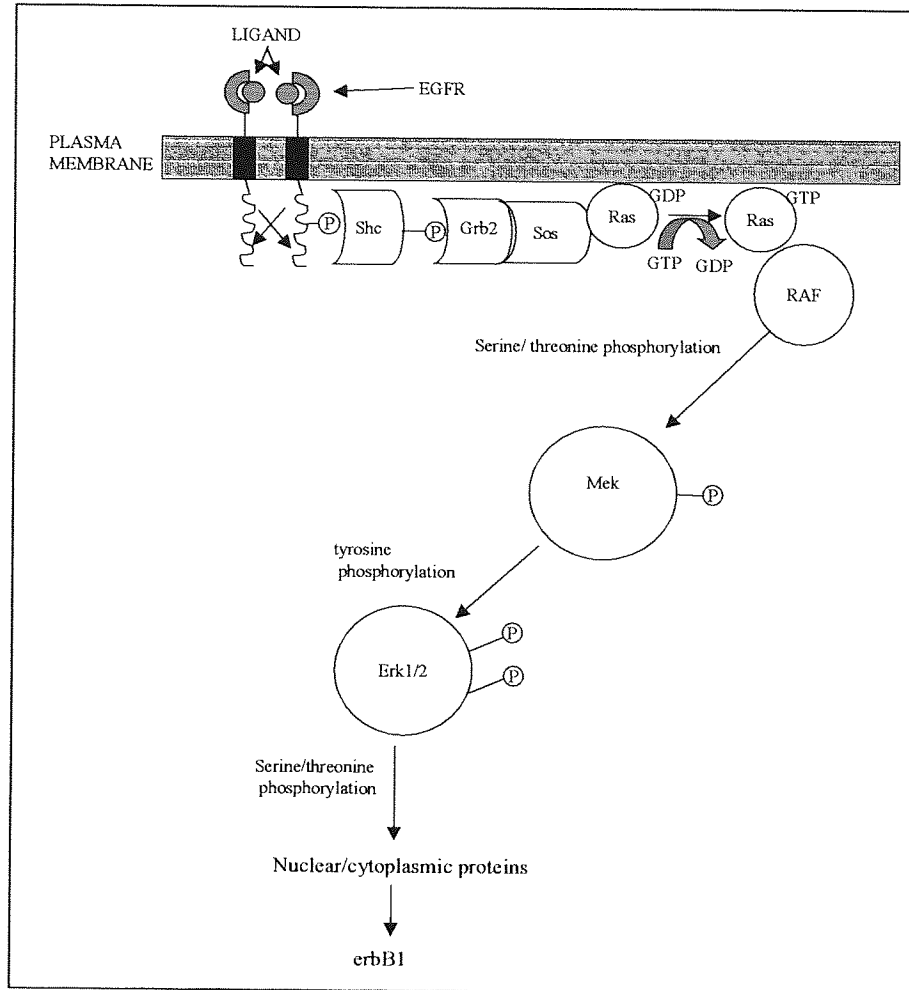
Figure 1.6: The typical pathway in which receptor tyrosine kinases allow transduction of a ligand initiated signal to be transmitted to the nucleus, upon where it can affect the growth characteristics of a cell by initiating gene transcription.

Upon autophosphorylation of the dimerised receptor tyrosine kinase domain, the tyrosine

residues are able to phosphorylate other molecules, in this case Shc. This molecule

attaches itself to the tyrosine domain of the receptor, allowing it to become acceptable for

binding by adapter molecules such as GRB2. GRB2 is responsible for the recruitment of

SOS, which is a RAS GDP-GTP exchange protein, *via* its SH3 domains. This allows SOS

to be recruited to the membrane to where it can interact with the membrane associated

RAS. SOS enables the activation of RAS by the transfer of GDP to GTP causing it to be

activated and able to further activate mitogen activated kinase kinase kinase (MAPKKK) molecules such as RAF (Janes *et al.*, 1994). RAF can subsequently phosphorylate MAPKK molecules such as MEK by phosphorylating their serine/ threonine residues, which in turn can phosphorylate MAPK molecules such as ERK1/2 by phosphorylating their tyrosine residues. Once the MAPK is active it can either activate cytoplasmic molecules or it can move into the nucleus and activate nuclear proteins by serine or threonine phosphorylation. These nuclear proteins can be transcription factors that when activated can bind to the DNA of the cell in specific regions to activate transcription of the DNA and thus other pathways that will initiate the cell to grow (Voet & Voet, 1995). Although this is a generalised pathway for many growth factors it can clearly be seen how EGFR can initiate a cell growth response by activating transcription of the necessary genes. Recent findings have shown that the above pathway has many cross-talk areas with other signalling pathways within the cell, particularly the lipid signalling pathways (Montgomery *et al.*, 1997) and the JAK-STAT pathway (Yamauchi *et al.*, 1997).

One of the striking characteristics of the *EGFR* (*ERBB1*) gene, located on chromosome 7, is its homology to the avian erythroblastosis virus oncogene (*v-ERBB1*), shown to induce malignancies in chickens. It has been shown that there is 97% sequence homology between the *EGFR* and *v-ERBB* tyrosine kinase domain (Ullrich *et al.*, 1984), and also that the *EGFR* gene (*c-ERBB1*), is found to be similar in sequence structure, although its mechanisms of cell proliferation induction are not clear (Haapasalo *et al.*, 1996; Haley *et al.*, 1987). The *v-ERBB* gene encodes for a truncated form of the receptor that lacks an extracellular ligand-binding domain. The same truncation has also been shown to occur with EGFR in humans and seems to lead to malignancies due to the constant activation of the tyrosine kinase domain and thus permanent activation of the proliferation pathways.

Malignancy due to EGFR misregulation can also be due to over-expression of the mRNA, which is often accompanied by co-expression of the EGF and TGF-α ligands. The over-expression of these ligands allows the tumour cell to stimulate growth in an autocrine manner, thus enabling it to survive and proliferate in an environment with fewer external growth stimuli (for review see Tang *et al.*, 1997).

Deletions of the *EGFR* gene result in a smaller protein product with three classes of deletions now found. Type I mutants lack the majority of the external domain including the ligand binding region and thus most closely resemble the *v-ERBB* oncogene, whereas type II mutants have external domain deletions next to the membrane that do not affect the ligand binding capabilities of the receptor. Type III are the most common type found in 17% of glioblastomas, in which they exhibit a deletion of 267 spanning amino acids in the domains I and II of the external region (Voldborg *et al.*, 1997).

In addition to glioblastomas, EGFR over-expression and amplification has been shown in a number of other malignancies, including breast cancers (Fox *et al.*, 1994), bladder cancer (Mellon *et al.*, 1996), and squamous epidermoid cancers (Ullrich *et al.*, 1984). Interestingly, evidence also suggests that patients who over-express EGFR have a poorer prognosis than those who do not (Khazaie *et al.*, 1993) and thus consequently therapeutic strategies that can potentially inhibit or reduce aberrant *EGFR* expression are of great use as anti-cancer agents.

1.9 Aims

The overall aim of this PhD thesis is to evaluate the use of DNA chip designed chemically modified oligodeoxynucleotides for the potential treatment of human malignant glioblastoma multiforme (GBM).

As stated GBM has no current effective form of treatment and it is hoped that the use of novel treatments such as antisense oligodeoxynucleotides, which can target specific problems within the malignant cell may hold the future for therapy for GBM. In this study the target of interest was that of EGFR gene which is greatly over-expressed in GBM (see sections 1.7).

The specific goals of this project that will be targeted to achieve this overall aim are:

1. To design and synthesise chemically stable oligodeoxynucleotides that can be delivered effectively to cells in a non-toxic way.

The major problem faced to date with antisense technology is ensuring that an antisense molecule can bind to its target, which thus has to be accessible within the cell. DNA chip-technology will therefore be used to select specific antisense sequences that can bind to the target molecule of interest, in this case the gene for the epidermal growth factor receptor (*c-ERBB1*). Once ODNs are synthesised, initial studies will be carried out with these sequences to determine whether they are biologically stable in their surroundings and deliverable to the target cell (Chapter 3).

2. *To ascertain the efficacy of initially designed oligodeoxynucleotides against EGFR protein and mRNA targets within an established cell line.*

Many of the problems with data that has been provided on antisense therapy so far has been the lack of specificity against the target, mainly due to inadequate design methods, and the lack of sufficient controls that prove that the oligodeoxynucleotides are acting in a sequence specific antisense manner. Studies will be performed to test the efficacy of antisense and control sequence oligodeoxynucleotides on cell proliferation and protein levels, both of the target and closely related sequences and of the mRNA levels. Modes of cell death will also be studied by FACS analysis (Chapter 4).

3. *To investigate the potential of glioma models to study antisense therapy and the potential use of combined therapies for the treatment of gliomas.*

Although the potential of antisense therapeutics is exciting, it is doubtful that one antisense molecule alone will be effective in reducing the malignant phenotype of any cancer. It is proposed that the future for antisense therapy would be to target either, multiple genes within the cell or combine the antisense treatment with that of conventional methods of treatment. Preliminary research will be undertaken to ascertain whether the combination of treatments would enable enhanced effects compared to those found with antisense alone. As *in vivo* studies cannot be undertaken, primary tumour cell lines will be established to enable testing of the antisense compounds as a potential therapy for gliomas (Chapter 5).

Chapter 2.0: Materials and Methods

General materials and methods used throughout this thesis are described in this chapter. Any minor changes for specific experiments are detailed in the individual chapters where they are used.

2.1 Design of Oligonucleotides

Required oligonucleotides (ODNs) were kindly designed by Dr. S Akhtar and Dr. M Sohail using DNA chip technology in the Department of Biochemistry at Oxford University. Briefly the technique was used to assess whether a variety of sizes of ODNs would be able to bind efficiently to the first 120 base pairs of the *EGFR* mRNA transcript *in vitro*. All combinations of ODN sequence from 1 to 21 bases were synthesised and immobilised on a synthetic glass support by amino linkages. *EGFR* mRNA was radiolabeled using ^{32}P and then walked along the ODN array upon which accessible sites showed hybridisation between the ODN and mRNA sequence. The array was then developed using phosphor imaging and the resultant image analysed by computer software to assess the exact sequences that had bound (for review see Southern, 1996).

This technique was used to determine sequences accessible within the initial 120 base pairs of the *EGFR* mRNA molecule and also to determine a sequence, which was not shown to have any binding efficiency as a control. These sequences were synthesised as phosphodiester, phosphorothioate and 2'-*O*-methyl derivatives for evaluation on cell growth, protein levels, mRNA levels and apoptosis.

2.2 RNA and DNA Synthesis

2.2.1 Synthesis of Oligonucleotides

Oligonucleotides were synthesised in the phosphodiester, phosphorothioate and 2'-*O*-methyl forms using an ABI 392 automated DNA/RNA synthesiser (Applied Biosystems, Warrington, U.K.) using standard phosphoramidite reagents (Cruachem Ltd., Glasgow, U.K.). The technique of automated solid phase synthesis was first used to synthesise polypeptides and was later adapted to perform the synthesis of oligonucleotides (Brown & Brown, 1991). The initial step of this method involves the coupling of the second required phosphoramidite base to a glass support, which contains the first oligonucleotide required. The dimer formed by the condensation reaction of the two nucleotides is protected by a dimethoxytrityl (DMT) group and any monomer reagent remaining is capped to prevent further polymerisation. The dimer formed is then deprotected to allow subsequent monomer bases to be attached which gives the production of a trityl group that is orange in colour and can be used to quantify the efficiency of the coupling reaction. When the desired sequence length is reached the oligonucleotide is cleaved from the support and deprotected with the addition of an ammonia solution. Synthesis occurs in the 3' to 5' direction to take advantage of the high reactivity of the 5' hydroxyl groups on the nucleotides.

2.2.1.1 *Phosphodiester Oligodeoxynucleotides*

Phosphodiester (PO) ODNs were synthesised on a 0.2μM scale using the pre-programmed 'CE cycle' (Cruachem Ltd., Glasgow, U.K.). The 5'-trityl groups were automatically cleaved during the synthesis and the final PO ODN was cleaved from its support with the addition of 1.5ml of ammonium hydroxide. PO ODNs were deprotected for 8 hours at 55°C to remove the protecting groups on the exocyclic amines.

2.2.1.2 *Phosphorothioate Oligodeoxynucleotides*

Phosphorothioate (PS) ODNs were synthesised using the pre-programmed 'sulphur' cycle on a 0.2μM scale with the sulphur groups being introduced by the use of tetraethyl disulphide (TETD) reagent (Applied Biosystems, Warrington, U.K.). The sulphurising agent replaces the oxidising reagent in the standard 'CE cycle' to allow the addition of the sulphur group instead of one of the non-bridging oxygens in the phosphate backbone. Synthesised PS-ODNs with 5'-trityl groups removed were cleaved with the addition of 1.5ml of ammonium hydroxide and were deprotected at 55°C for 8 hours to remove the remaining base protecting groups.

2.2.1.3 *2' O-methyl Oligonucleotides*

Oligoribonucleotides consisting of 2'-*O*-methyl bases either completely or as a chimeric sequence with PS bases were synthesised using the standard RNA cycle on a 0.2μM scale using 2'-*O*-methyl phosphoramidite bases. Where chimeric ODNs were synthesised the

66

sequence was synthesised in separate parts with the last base of the first 3' to 5' sequence being the first base of the next part of the sequence and subsequently therefore having its final DMT left on and the cleavage from the column only occurring after the entire sequence was synthesised. Both normal and chimeric sequences containing 2'-O-methyl bases were cleaved using 1.5ml of ammonium hydroxide and deprotected at 55°C for 8 hours.

2.2.2 Coupling Efficiency Evaluation

During synthesis of all ODNs used the first two and last two DMT groups were collected in order to analyse coupling efficiency of one base to the next by spectrophotometry. When the trityl group is cleaved it exists as a cation, which produces an orange colour in acid conditions. Each trityl fraction is diluted to 25ml with 0.1M p-toluene sulphonic acid in acetonitrile. The absorbance of the solutions were then measured in UV-visible spectrophotometer at 495nm (Jenway Scientific Instruments, U.K.) and the yield was calculated using the following formula (Brown & Brown, 1991):

Overall % yield = last or lowest fraction / second or highest fraction x 100

Stepwise yield = overall yield $^{(1/no. of couplings)}$

% stepwise yield (coupling efficiency) = stepwise yield x 100

The first trityl fraction was not used to determine coupling efficiency as this represents the first base attachment to the column. A percentage stepwise yield of 97-99% was expected for ODN synthesis performed and only ODNs of this yield were used in subsequent experiments.

2.2.3 Purification of Oligonucleotides

Following deprotection of all ODNs used they were purified to remove any uncoupled bases and free salts remaining. ODNs were purified through sephadex G-25 packed (NAP-10) columns (Pharmacia Biotech, St Albans, U.K.) using gravity separation for maximum product recovery. The columns were washed through with 15ml of DNA/RNA-free water and then the ODN was added in a total of 1ml. Once the ODN had passed completely into the beads, a further 1.5ml of water was added to elute the ODN in a total volume of 1.5ml collected into a microcentrifuge tube. The purified sample was then dried by centrifugation using a Savant DNA Speed Vac (Savant, U.K.) and stored until required at $-20°C$.

2.2.4 Quantification of Oligonucleotides

As the purine and pyrimidine bases of RNA and DNA absorb light at a wavelength of 260nm they can be quantified spectrophotometrically. The following method converts OD units into micromolar concentration (Brown & Brown, 1991).

2.2.4.1 Estimation of the Molecular Weight

For unmodified PO ODNs:

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

Where: nA = number of adenine bases in the sequence and n = total number of bases.

(64 x n-1) accounts for the molecular weight of the phosphate groups

This method however does not account for the differences in molecular weight when using phosphorothioate and 2'-O-methyl additions to the backbone and bases. The following adjustments can be made to account for these differences.

For phosphorothioates (PS) a sulphur atom is added to the phosphodiester side chain in place of an oxygen atom. Thus there is an adjustment of +16 as the molecular weight of the atoms changes from 16 to 32. This +16 is added to the n-1 amount in the molecular weight calculation.

i.e. MWt = (249 x nA) + (240 x nT) + (265 x nC) + (225 x nG) + (**80** x n-1) +2

For 2'-O-methyl modifications an oxymethyl group (OCH_3) is added to the 2'-carbon of the sugar moiety thus giving an increase from 1 (assigned previously to the hydrogen atom in that position) to 31, thus an addition of 30 for each base that contains the modification. There is also an allowance to account for the molecular weight difference between thymidine and uracil (MWt = 226).

2.2.4.2 *Estimation of the Molar Extinction Coefficient, ε at 264nm*

ε = {(15.4 x nA) + (8.8 x nT) + (7.3 x nC) + (11.7 x nG)} x 0.9*

*It is necessary to multiply the extinction coefficient of the sum of the individual bases by 0.9 to account for the stacking interactions in the single strand as they suppress the absorbance of DNA at 260nm.

2.2.4.3 *Conversion of OD Units to Molar Concentration*

grams of ODN for 1 OD unit = ε_{ODN}/ MWt$_{ODN}$

grams of ODN x OD = grams of ODN in sample volume used for OD measurement

grams of ODN in sample volume/ MWt = mmoles of ODN in sample volume

mmoles ODN in sample volume/ sample volume = molar concentration of ODN

2.3 Labelling of Oligonucleotides

2.3.1 5'-end $[^{32}P]$ –Radiolabelling

The synthesis of ODNs allows the 5' termini to be absent from a phosphate group thus allowing easy transfer of a phosphate to the 5' end from a suitable donor. The γ-^{32}P from $[\gamma$-^{32}P$]$-ATP can be easily transferred to the 5' termini using the enzyme bacteriophage T4 polynucleotide kinase (PNK) (Sambrook *et al.*, 1989).

$$DNA_{OH}^{5'} \text{ or } RNA_{OH}^{5'} \xrightarrow{\text{T4 PNK}} 5'[^{32}P] \text{ DNA or } 5'[^{32}P] \text{ RNA } + ADP$$

$$[\gamma\text{-}^{32}P]ATP$$
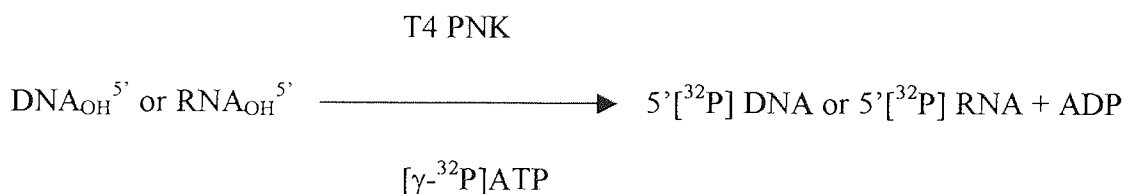
The ODNs were labelled at the 5'-end with [γ-^{32}P] labelled ATP (ICN, U.K.) using

bacteriophage T4 PNK (Bioline, U.K.) in a 1x forward reaction buffer (100mM Tris pH

7.5, 20mM MgCl$_2$, 10mM DTT, 0.2mM spermidine and 0.2mM EDTA) at 37°C for 45

minutes. The general reaction mixture is given below:

ODN (100 pmoles)	1μl*
T4 kinase (5units / μl)	1μl
10x reaction buffer	2μl
[γ-^{32}P] dATP (4500Ci / mmol)	1μl
sterile DEPC-treated ddH2O to	20μl

* volume of ODN varied depending on concentration of stock solution

2.3.2 Purification of Labelled Oligonucleotides

Once labelled the ODNs were purified to remove any free label and reagents left over

from the reaction. Radiolabeled ODNs were purified by separation on 20% PAGE gels as

detailed in section 2.4.1 to remove any free label remaining in the reaction solution and to

remove any residual reagents. Samples were run on the gel until the loading dye front had

reached the end of the gel, and then the position of the bands were visualised by exposure

to autoradiographic film (section 2.4.2). Bands containing the required ODN were cut

from the gel and placed in a 15ml centrifuge tube (Sarstedt, U.K.) along with 3mls of

ddH2O and eluted overnight on a shaker at room temperature. The water containing the

ODN was removed, transferred to a sterile microcentrifuge tube and lyophilised (Savant

DNA Speed Vac) and the resultant purified pellet was stored at –20°C until required.

2.4 General Analytical Methods

2.4.1 Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gel electrophoresis is a method readily used for the size separation of short chain nucleic acids such as ODNs. The gels are formed by the polymerisation of acrylamide monomers (CH_2=CH-CO-NH_2) into random polyacrylamide chains cross-linked by a co-monomer, N-N'-methylene-bis-acrylamide, mostly known as "Bis-acrylamide". The resulting gel can have varying pore sizes to aid in size separation determined by the concentrations of both acrylamide and Bis-acrylamide.

Polyacrylamide gels were prepared as stated in Sambrook *et al.*, (1989) using a Biorad Protean II electrophoresis rig. Gels were cast between two glass plates (20cm x 20cm), which had been cleaned using water, ethanol and finally acetone. The plates were separated using 1mm spacers and once the gel was poured a 1mm comb was inserted containing 15 wells for sample loading. Polymerisation of the gel mix was activated by the addition of 600ul of fresh 10%(w/v) ammonium persulphate (AMPS) and 40ul of TEMED to a total of 50ml of a stock gel mix of the percentage required. In general ODNs were separated using a 20% polyacrylamide gel mix and RNA was separated on a 6% polyacrylamide gel mix with details of the specific gel make up in relevant sections. Gels were left to polymerise for a minimum of 20 minutes before wells were washed with sterile 1x TBE, diluted from 10x TBE stock solution (108g Tris Base, 55g Boric Acid, 20ml 0.5M EDTA (pH8.0) made up to 1 litre with ddH$_2$O). Samples were diluted in relevant loading buffers and loaded into wells using round-ended micropipettes (Costar,

U.K.). During running of the gels the system is cooled to 10°C using a thermostat controlled water circulator (Sarver Instruments, U.K.)

2.4.2 Autoradiography

2.4.2.1 Sample Detection

Radiolabeled samples separated by PAGE were detected by autoradiography. Gels were removed from the glass plate wrapped in a single layer of Saran Wrap (Fischer Scientific, U.K.) and placed face up in an autoradiograph cassette fitted with an intensifying screen (Amersham Life Sciences, Amersham, U.K.). The gel was exposed to a sheet of Kodak HP autoradiograph film (Amersham Life Sciences, Amersham, U.K.) under dark room conditions between 20 seconds and 48 hours depending on the intensity of the signal. For particularly weak signals the cassette was stored at −70°C to enhance the intensifying screen and then thawed before developing. All films were developed and fixed using Kodak photographic reagents (Sigma, U.K.) before being allowed to dry naturally.

2.4.2.2 Densitometric Analysis of Autoradiographic Images

Autoradiographs were scanned using a Trust SCSI Connect scanner connected to a PC computer and images were saved as uncompressed TIFF files. Scanned images were then analysed using NIH imager v1.58 program (Division of Computing and Research Technology, NIH, Bethesda, USA), which allowed plotting, and quantification of the relevant image intensities of band patterns on the autoradiographs.

2.4.3 Liquid Scintillation Counting

Liquid scintillation counting (LSC) was used to quantify the activity of $[^{32}P]$ radiolabeled nucleotides. Samples of nucleic acids were added to 5ml of Optisafe Hisafe III (Pharmacia-Wallace, St-Albans, U.K.) and counted for 5 minutes using a Packard 1900TR Scintillation Counter. Adjustments were made for background radioactivity levels and for decay during the experimental procedure by entering half-life and reference data of the isotope used. Comparing counts obtained for each sample to those obtained for background enabled an analysis of radioactivity present in individual samples.

2.5 Stability Studies of ODNs in Media

Radiolabeled ODNs were incubated in 300μl of DMEM media containing serum or serum-free media at 37°C for up to 48 hours. Sufficient ODN was added to give a minimum activity of 10 cpm in each 20μl sample taken at each time point during the experiment. Removed samples were mixed with a loading buffer containing 9:1 formamide: 1X TBE, 0.25% bromophenol blue, 0.25% xylene cyanol and frozen at – 20°C prior to loading on a gel. A 20% polyacrylamide denaturing (7M urea) gel mix (33ml 30% acrylamide / bis-acrylamide (29:1), 4.2g urea, 5ml 10x TBE, 12ml ddH₂O) was used to analyse the degradation profile of the ODN before autoradiography (section 2.4.2).

2.6 Cell Culture Techniques

All cell culture procedures were performed in aseptic conditions in a Gelaire, Biohazard Level II laminar flow hood from ICN (Thame, Oxfordshire, U.K.)

2.6.1 Cell Lines

The main cell line used was A431, a vulval carcinoma derived line which expresses the EGFR protein at levels 10 to 50 fold higher than seen in other cell lines (Ullrich *et al*, 1984). These cells were a generous gift from Dr P.L.Nicklin, Ciba Geigy Pharmaceuticals (Horsham, U.K.). Two primary cell lines were also used that were kindly donated to the Department of Neurological Surgery at the Institute of Neurology, London with patients consent. Patients were diagnosed and treated for malignant gliomas and upon surgical removal of the tumour a small sample was used to establish a cell suspension and subsequent culture for experimental purposes. The two cell lines established were termed IN2045 and IN859.

2.6.2 Culture Media

A431 cells were grown in Dulbecco's Modified Eagles Media (DMEM), containing 10% v/v mycoplasma screened foetal bovine serum (FBS), 2mM L-glutamine and 1% penicillin/streptomycin (all supplied by GIBCO BRL, Paisley, U.K.). For certain studies media was required to be serum free and thus the above media was used without the addition of FBS.

Media for IN2045 and IN859 was HAMS F-12 (GIBCO BRL, Paisley, U.K.) supplemented with 1mM glutamine and 10% v/v mycoplasma screened FBS.

2.6.3 Maintenance of Culture Stocks

General stocks of A431 cells were seeded into 75cm^2 vented cap tissue culture flasks with a 0.2μM hydrophobic micro-porous membrane vent (Costar, U.K) containing approximately 25ml of complete DMEM media. The flasks were placed in a 95% humidified incubator with an atmosphere of 5% CO_2 in air and at a temperature of 37°C.

Stock cultures were maintained by passaging every four days or when near confluence, with media changes every 48 hours to replenish nutrients. The cells were passaged using standard aseptic procedures as briefly described below.

The spent media was aspirated and the adhered cells were washed with excess (approximately 10ml) of phosphate buffered saline solution (PBS) (Sigma, Poole, U.K). Following aspiration of the PBS, 3ml of 1X Trypsin/EDTA (0.25% w/v trypsin, 0.2% disodium ethylenediamine tetraacetate (EDTA) in PBS, pH 7.2) (GIBCO BRL), was added and the flasks incubated at 37°C for 5 mins or until the cells became detached from the flask. The trypsin was neutralised by adding 5mls of fresh complete media and the cells agitated to ensure that they were singular. Finally the cells were placed in a 15ml centrifuge tube (Costar, U.K) and centrifuged at 1000 rpm for 5 minutes (Mistral 3000 I centrifuge, Sanyo MSE, Leicester, U.K.) to obtain a cell pellet. The media was aspirated and the pellet was then resuspended in complete media. The cells were reseeded into a fresh flask to give the required density in a total volume of 25ml of fresh media. In

general cells were split at a ratio of 1:10 for general stocks but for specific experimental seeding they were counted by haemocytometer and transferred at the required cell number to the appropriate cell culture vessel.

Primary cell lines were maintained in much the same way but Hanks balanced salt solution (Sigma, Poole, U.K) was used to wash the cells in place of PBS. Cells were reseeded at a 1:5 ratio in this case as their growth rate differed from that of A431 cells.

2.6.4 Freezing and Thawing of Cell Lines

When dealing with cell lines it is always important to keep a reserve frozen culture stock that can be thawed when necessary to replenish lost stocks. The protocol for freezing and thawing of all cell lines was as follows.

2.6.4.1 Freezing

Stock cells were trypsinised as above, counted (see section 2.6.5) and pelleted by centrifugation at 1000rpm for 5 minutes. The pellet was resuspended in freezing media (90% FBS and 10% DMSO (Sigma, Poole, U.K)) to a final cell density of greater than 1 x 10^6 cells ml^{-1}. A 1ml aliquot of the cells was then placed in a 2ml screw top cryovial (Costar, U.K), which was subsequently placed at -70°C overnight, before placing into a liquid nitrogen cell bank (-196°C) for long-term storage.

2.6.4.2 Thawing

Cells were recovered from nitrogen by rapid thawing at 37°C and gradual resuspension in their appropriate media, before being placed into 25cm^2 vented cap flask. Once cells had reached sufficient confluence in these flasks they were placed in 75cm^2 flasks and routinely maintained as in section 2.6.3.

2.6.5 Determination of Cell Number and Viability

As well as counting the cells of the stock cultures to determine cell density the viability of the cells can also be determined. It is important to establish viability to ensure that the cells are healthy, especially when they are initially thawed out as they can be damaged by the freezing media and die due to shock. Viability was assayed using the trypan blue exclusion test in which 100µl of trypan blue (4mg ml^{-1}) (Sigma, U.K) was mixed with 400µl of cell suspension. A small amount of the trypan blue cell suspension was then transferred to the counting chamber of a Neubauer Haemocytometer with depth of 0.1mm and area 1/400mm^2 (Weber Scientific International Ltd, U.K). The cells were counted in the four corner squares (1mm^2) of the haemocytometer using a light microscope and the mean count *per* square obtained. Since live cells do not take up the dye, while dead cells do, then unstained (viable) and stained (non-viable) cells could be counted. The cell density was determined using the following equation:

Viable cells *per* ml = average count *per* square x 10^4 x dilution factor of trypan blue

The percentage viability can be determined as follows:

% cell viability = viable cells (unstained) / total number of cells (stained and unstained)

Cell suspensions with less than 95% viability were discarded and not used.

2.7 Cell Association Studies

A431 cells were used for studies to determine the association of ODN with cells in the presence and absence of cationic lipids and at varying temperatures. Cells were cultured on 24 well tissue culture plastic plates (Costar, U.K.). Sub-confluent stock cultures were harvested and counted as described in section 2.6.3 – 2.6.5 and the cell density diluted to 5×10^4 cells ml^{-1} with DMEM media. Each well was seeded with 1ml of suspension (5 x 10^4 cells) and incubated at 37°C in a humidified incubator overnight. The following day, the cells had reached about 80-90% confluency and were used for association experiments as briefly described below and depicted in figure 2.1.

The plating media was aspirated and the cell monolayer briefly washed twice with 1ml of PBS at 37°C to remove any remaining serum and media. The PBS was aspirated and replaced with 200μl of warmed serum-free DMEM media containing radiolabeled ODN and lipid at the required concentration. Cells were incubated for 4 hours at either 4°C or 37°C. Following incubation apical media was collected and counted by liquid scintillation counting (LSC) a described in section 2.4.3. Cells were then washed 5 times with 1ml of ice cold PBS-azide to inhibit any further metabolism and to remove and loosely associated ODN and lipid. Washings were collected and radioactivity determined by LSC. Cell monolayers were then solubilised by agitation in the presence of 0.5 ml 3% v/v

Triton X-100 (Aldrich Chemical Company, Gillingham, U.K.) in distilled water for 30 minutes at 37°C. Cells were washed twice more with 0.5 ml of Triton X-100 to ensure that all cells and thus radioactivity had been removed. Cellular radioactive content was determined by LSC.

For all association experiments 3 extra wells were seeded with cells for determination of cell number and viability upon harvesting. This allowed normalisation of cell number between multiple experiments and to ensure viability of cells and thus results.
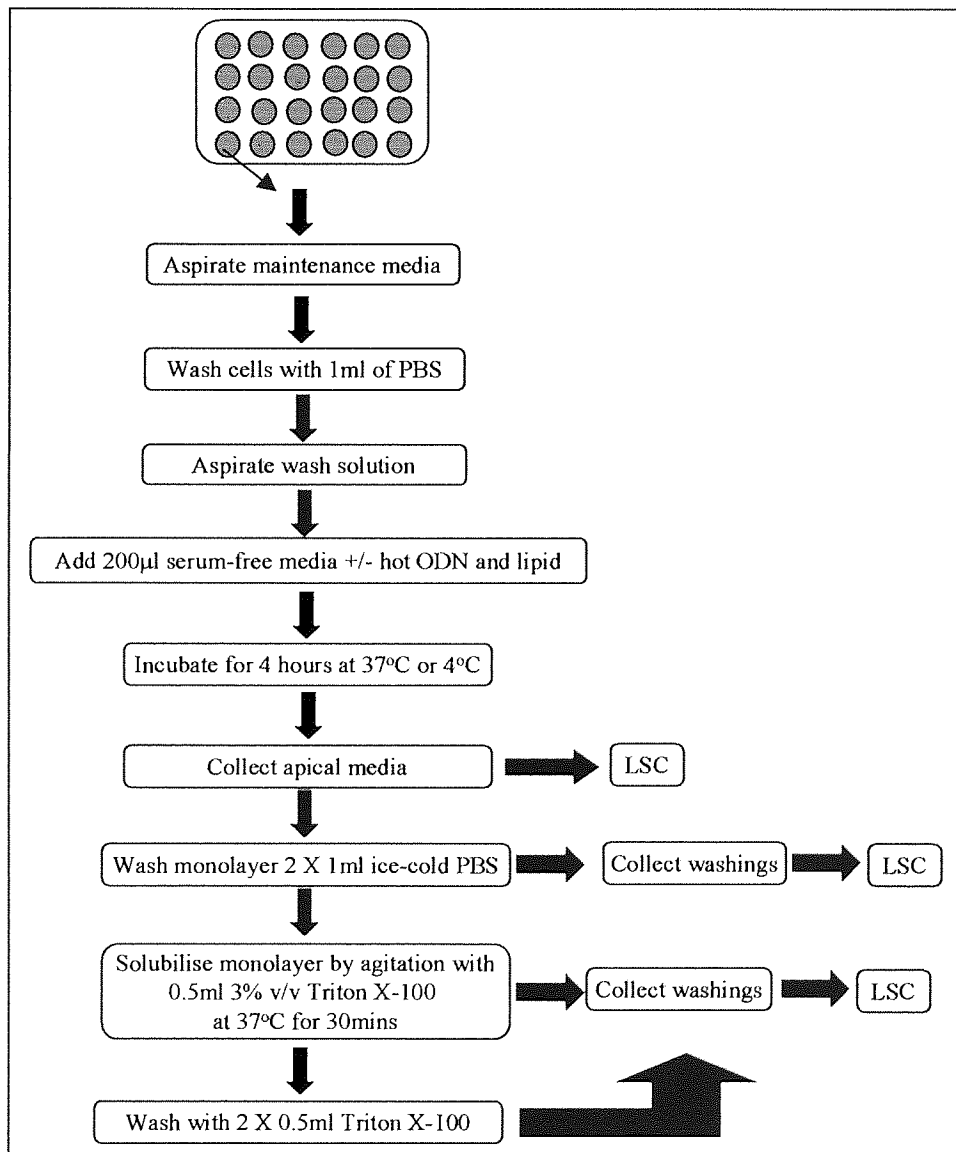
Figure 2.1 Schematic representation of generalised protocol for cell association experiments.

2.8 Efficacy Studies

In order to determine the biological efficacy of the various ODNs designed, experiments were conducted to determine the effect of ODN on cell number and thus proliferation of the cells.

2.8.1 Cell Number Determination

2.8.1.1 Trypan Blue Exclusion

Cells were seeded into 24 well plates (2cm^2) (Costar, U.K.) in complete media at 5 x 10^4 cells / well in a total volume of 1ml and left to settle for 20-24 hours in a humidified incubator. After washing monolayers with 1ml of PBS, ODNs alone or in complex with lipid were added at the desired concentration in a total volume of 200μl serum-free media. Treatments were added for 4 hours after which time the media was aspirated and replaced with fresh serum-free media for the rest of the experiment.

After incubating for the desired time period the media was aspirated and the cells were washed with 1ml of PBS, trypsinised and the number of viable cells counted by trypan blue exclusion as described in section 2.6.5. In order to determine any change in the morphology of the cells after treatment their morphology was examined by light microscopy and compared to untreated cells.

Crystal violet is a basic dye that can bind to any negatively charged molecules in the cell (i.e. proteins and DNA) and thus stains the cells purple. When cells proliferate they increase the amount of proteins and DNA in the cell and thus an increase in the amount of staining represents an increase in cell number. Measuring the absorbance of the cells using densitometry provides a quick and simple method for quantifying cell number / growth (Fell, 1999).

A431 cells were plated out into 96 well plates ($0.3cm^2$) (Costar, U.K) at a concentration of 5×10^3 cells / well in a total volume of 100µl complete media and the ODN added at various concentrations with up to four replicates per concentration. The cells were then left for 20-24 hours in order for them to settle and begin to grow. The growth media was aspirated and the cell monolayer briefly washed with 100µl warmed PBS, which was then also aspirated. ODNs with or without lipids were added to the cells at the required concentration and left for 4 hours in serum-free media after which the media was aspirated and replaced with fresh serum-free media. Cell monolayers were washed with PBS-sucrose three times, by removing 50µl of media and adding 50µl of wash solution. Cells were fixed by adding 50µl of fix solution (4% paraformaldehyde in wash solution) and left for 30 minutes. After this period the entire well contents were removed and 100µl of fix solution was added and incubated for a further 1 hour. After fixing cells were washed with excess PBS three times, after which the plate was emptied and blotted dry. Cells were stained using 50µl per well of crystal violet (0.1% in PBS) (Sigma, Poole, U.K.) and left to be taken up by the cells for 30 minutes after which it was thoroughly washed by submersing the entire plate in distilled water five times. After drying the

stained cells were solubilised by the addition of 50µl of 1% SDS in water (BDH, U.K) for

10 minutes. The plate was read on an automatic plate reader at 550nm and the results

analysed. A standard curve, correlating cell number to absorbance, was obtained by

measuring the absorbance (OD_{550}) of replicate wells containing known numbers of cells

ranging from (1×10^3 to 1×10^5 cells / well). Cells were plated in 96 well plates in 100µl

of complete media and left to attach for 4 hours. The crystal violet assay was then

performed as stated above. Cell number was plotted against OD_{550} and the regression

equation calculated was used to convert OD_{550} of experimental samples to cell number.

2.9 Protein Analysis

In order to analyse any changes in protein levels in the cells caused by treatment with

ODN and / or lipid the cells had to be lysed in a way that would release the protein with

minimal disruption of the proteins. Once isolated the proteins can be denatured using a

strong anionic detergent such as SDS or Triton X-100 with the addition of heat and a

reducing agent to allow protein dissociation. Denatured polypeptides can bind to

negatively charged SDS enabling separation through gel electrophoresis according to size

(Sambrook *et al.*, 1989).

2.9.1 Protein Lysate Preparation

Cells required for protein analysis were seeded into 24 well plates at 5×10^4 cells / well

in a total volume of 1ml of complete media. Cells were left overnight to settle and treated

the following day with ODN and lipid for 4 hours in serum-free media after which the

media was replaced with fresh serum-free media for a further 20 hours. Treatments were performed with one dose over 24 hours or 2 doses over 48 hours in a total of 8 wells per dose / ODN. After treatment cells were washed with 1ml / well of PBS and trypsinised using 2 x 150μl of trypsin as in section 2.6.3. The replicate wells were pooled together and centrifuged at 1000rpm for 5 minutes at 4°C to pellet the cells. Cell pellets were washed twice more with PBS in this manner and the cells counted on the final wash by trypan blue exclusion (section 2.6.5). Cells were lysed by the addition of 100μl lysis buffer (0.5M Tris-HCl, pH6.8; 10% glycerol; 10% Triton X-100; 0.1mM Leupeptin and 0.1mM PMSF all from Sigma, Poole, U.K.) for every 1×10^6 cells. Cell lysates were vortexed and sonicated on ice until the solution was evenly mixed and then centrifuged at 14000rpm for 30 minutes at 4°C. To the supernatants 5X Laemelli buffer (367mM Tris-Base (pH 6.8), 5.7% SDS, 5.7% β-mercaptoethanol (14.6M), 28.7% Glycerol, 0.2% bromophenol blue) was added before storage at –20°C until required for analysis.

2.9.2 Protein Determination

The Bio-Rad protein assay was used to determine the amount of total protein in whole cell lysates prepared as described in section 2.9.1 and is based on the colour change of a dye in response to change in the amount of protein in a sample and is known as the Bradford method. The absorbance maxima for an acidic solution of Coomassie Blue R-250 shifts from 465nm to 595nm when it binds to proteins and thus the greater the amount of protein will give a larger absorbance reading.

A standard curve of protein concentration was first prepared using 1mg/ml of BSA diluted with distilled water to a final volume of 800μl (containing 5μl of lysis buffer)

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giving a range of concentrations from 0μg/ml - 25μg/ml. Whole cell lysate samples were also analysed by adding 5μl of sample in duplicate to 795μl distilled water. Both standards and samples were then thoroughly mixed with 200μl of Bio-Rad reagent and incubated at room temperature for 15 minutes. Absorbance at 595nm was then read using a spectrophotometer (Jenway, U.K.) and subtracted from a blank control (0μg/ml protein) and a calibration curve was constructed of absorbance versus concentration (μg/ml) of the protein standards at 595nm. The calibration curve was then used to determine the amount of protein in the unknown cell lysates samples.

2.9.3 SDS Polyacrylamide Gel Electrophoresis

All protein electrophoresis was carried out using Bio-Rad electrophoresis apparatus (Bio-Rad, California, USA). Two glass plates (7cm x 8cm) were assembled in a clamp stand with spacers (1.5mm thick) at the sides to form a gel sandwich sealed at the base. A 7.5% polyacrylamide resolving gel was prepared as described in table 2.1 with the TEMED being the final addition to initiate gel polymerisation and then poured between the gel plates to approximately 2cm below the top of the plates. The gel was overlaid with 200μl of water-saturated isobutanol (Sigma, Poole, U.K.) and left to polymerise for 30 minutes. After polymerisation the isobutanol was washed off with excess distilled water and between the plates dried with filter paper to remove any remnants of alcohol. A 4% stacking gel was prepared, as shown in table 2.2. and poured on top of the resolving gel. A 10 well comb was inserted into the stacking gel and the gel was left to polymerise for a further 20 minutes. The comb was then removed and the wells washed with distilled

water before the whole assembly was placed in a gel tank with both the lower and upper

chambers filled with running buffer (0.3% Tris-Base, 1.44% glycine, 0.1% SDS).

STOCK / REAGENT	VOLUME	FINAL CONCENTRATION
30% acrylamide/ bis mix (29:1)	2.5ml	7.5%
1.5M Tris-HCl (pH8.8)	2.5ml	0.375M
Distilled Water	4.8ml	N/A
10% (w/v) SDS	0.1ml	0.1%
10% (w/v) fresh AMPS	0.1ml	0.1%
TEMED	10μl	N/A

Table 2.1: Composition of 7.5% resolving gel mix

STOCK / REAGENT	VOLUME	FINAL CONCENTRATION
30% acrylamide/bis mix (29:1)	650μl	4.0%
1.5M Tris-HCl (pH6.8)	1.25ml	0.125M
Distilled Water	3.0ml	N/A
10% (w/v) SDS	50μl	0.1%
10% (w/v) fresh AMPS	50μl	0.1%
TEMED	10μl	N/A

Table 2.2: Composition of 4% stacking gel mix

Protein samples were heated to 95°C for 5 minutes and then immediately loaded into the

pre-formed wells using round-ended micropipette tips (Costar, U.K.). As well as the

samples a pre-stained high range molecular weight marker of known molecular mass

(45kDa – 205kDa) (Sigma, Poole, U.K.) was also loaded into one well of each gel. The

coloured bands were visualised both on the gel and after transfer to nitrocellulose

membrane allowing comparison with sample protein bands to allow size estimation of

samples. The gel was run at a constant ampage of 40mA per gel for approximately 40

minutes or until the bromophenol blue dye had ran off the bottom of the gel.

2.9.4 Electrophoretic Transfer of Separated Proteins

Following gel separation the proteins were contained within the resolving gel and so the stacking gel was discarded. The proteins were transferred to nitrocellulose Hybond-ECL membrane (Amersham Life Sciences, Buckinghamshire, U.K.) by the use of a Bio-Rad Trans-Blot electrophoresis transfer cell. The nitrocellulose along with 6 sheets of filter paper (Whatman 3MM, U.K.) were cut to the size of the gel and along with the gel were wetted in transfer buffer (25mM Tris-Base, 192mM glycine and 20% w/v methanol) prior to blotting. A blotting sandwich was assembled consisting of three sheets of filter paper, nitrocellulose, gel and a final three sheets of filter paper and was placed in the transfer apparatus as shown in figure 2.2 below. Any air bubbles were carefully removed from the sandwich to ensure efficient transfer and the tank was filled with transfer buffer so that the sandwich was completely submerged. Transfer was performed either at 215mA for 1.5 hours or at 30V overnight.
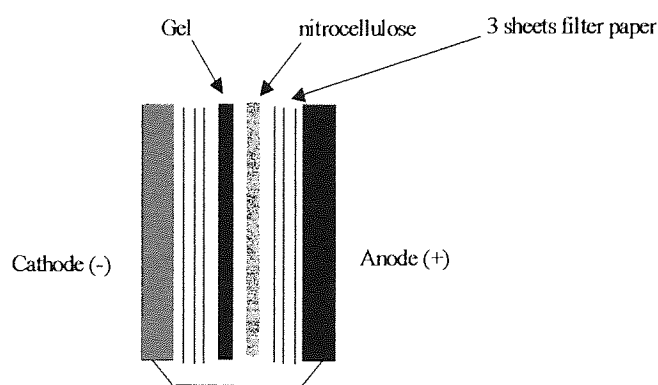
Figure 2.2: Representation of assembly of blotting sandwich for electrophoretic transfer of proteins.

After completion of blotting the assembly was unassembled and the nitrocellulose processed for immunodetection and ECL analysis as described in section 2.9.5.

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2.9.5 Immunodetection and Analysis

Following blotting, the nitrocellulose membrane containing the transferred proteins, was subjected to antibody treatments and immunodetection to allow visualisation of the specific proteins of interest.

Following blotting the nitrocellulose filter was incubated in blocking buffer (5% w/v non-fat dried powdered milk dissolved in PBS-Tween (1% Tween 20 in PBS pH7.5)) for one and a half hours at room temperature. The blots were then washed in PBS-Tween (2 x 50mls x 10min) and then incubated for 1 hour in the appropriate primary antibody in 6ml of PBS-Tween. Primary antibodies used were mouse anti-EGFR E-3138 (Sigma, Poole, U.K.) at a dilution of 1 in 1000, rabbit anti-actin A2066 (Sigma, Poole, U.K.) at a dilution of 1 in 400 and mouse anti-ErbB2 E19420 (Signal Transduction Laboratories, USA) at a dilution of 1 in 2500. Following primary antibody treatment the membrane was washed in PBS-Tween (2 x 50ml x 10min) before the addition of the HRP-conjugated secondary antibody, either IgG anti-mouse (NA931) or IgG anti-rabbit (NA934) (both Amersham Life Sciences, Buckinghamshire, U.K.). Secondary antibodies were diluted 1 in 2500 in 6ml of PBS-Tween and incubated for one hour. Membranes were washed again in PBS-Tween (2 x 50ml x 10min) before ECL detection in the dark room. Equal volumes of ECL reagents 1 and 2 (Amersham Life Sciences, Buckinghamshire, U.K.) were mixed together (final volume $0.125ml/cm^2$), applied to the blot for one minute and then excess was removed before the blot was wrapped in Saran wrap cling film and placed in an autoradiograph cassette. The membrane was exposed to a piece of autoradiograph film for 20secs – 1 min after which the film was developed until bands appeared, rinsed in water and then fixed and air dried. Repeat exposures were undertaken to achieve an

optimal image before densitometric analysis as descried in section 2.4.2.2. When two proteins were to be analysed on the same gel (i.e. EGFR and actin) the blot was stored at 4°C overnight to allow degradation of the signal and the whole procedure repeated again, including blocking.

2.10 RNA Analysis

In order to determine the effect of antisense ODNs on the *EGFR* mRNA levels of A431 cells the RNA has to be extracted from the cell, purified and then analysed. Two methods, RT-PCR and RPA analysis were used to assess the effect of ODN on mRNA levels in order to provide an accurate analysis of any effect found.

2.10.1 RNA Extraction

A431 cells were plated in 24 well plates at 5×10^4 cells/well in a total of 1ml of media and left to settle overnight. Cells were then treated with ODN and lipid as required for 4 hours in serum-free media and the media then replaced with fresh serum-free media for a further 20 hours. Treatment was then repeated again for a further 24 hours giving a final two doses over 48 hours with a total of 8 wells *per* dose / ODN required. Following treatment the cells were washed with 1ml / well of PBS and trypsinised using $2 \times 150\mu l$ of trypsin as in section 2.6.3. The replicate wells were pooled together and centrifuged at 1000rpm for 5 minutes at 4°C to pellet the cells. Cell pellets were washed once more with PBS in this manner and the cells counted by trypan blue exclusion (section 2.6.5). RNA was then extracted using an SV Total RNA Isolation System (Promega, USA).

Briefly the cells were lysed using a lysis buffer containing β-mercaptoethanol and gaunidium thiocyanate to disrupt the cells and inactivate ribonucleases. Solubilised RNA was separated from other cell debris by heating and centrifugation and then precipitated with ethanol before attachment to the silica surface of a spin column. After washing the attached RNA is treated with DNase 1 to remove any DNA that will also attach to the column, before further purification by washings to remove any remaining salts and cellular impurities. RNA is finally eluted from the column using nuclease–free water. Purity of extracted RNA was assessed by the ratio of absorbances at 260nm and 280nm. A ratio of 1.7-2.0 was determined to be pure.

2.10.2 RT-PCR Analysis of mRNA Levels

This procedure allows the amplification of part of the *EGFR* sequence from the extracted RNA so that the fragment of interest can be resolved on an agarose gel to determine any changes in the *EGFR* mRNA levels following ODN treatment. There are a wide variety of kits available on the market that allow analysis of mRNA changes by RT-PCR and in this case a one-step kit was used, the Access RT-PCR System (Promega, USA) with minor variations as described below. The basic principles of the RT-PCR reaction are in two stages the reverse transcription of the RNA to cDNA and then amplification of the required cDNA fragment to give a final product that can be visualised on an agarose gel.

For optimisation of the procedure specific for the primers designed, $MgSO_4$ and primer concentration had to be determined by performing a concentration curve. Dr Marcus Hughes optimised these conditions within the laboratory. Two products were analysed in

all RT-PCR reactions from the same reaction, *EGFR* and actin. The following conditions were used for the reactions.

REAGENT	FINAL CONCENTRATION
5X Buffer	1X
$MgSO_4$	2mM
dNTPs	$2\mu M$
EGFR primer 1	$1\mu M$
EGFR primer 2	$1\mu M$
Actin primer 1	$0.2\mu M$
Actin primer 2	$0.2\mu M$
AMV	5 units
Tfl	5 units
RNA	0.5ug
dH_2O	Final volume of $50\mu ls$

Table 2.3: Reaction mixture for all RT-PCR reactions.

Conditions of the RT-PCR were as follows:

48°C 45min

94°C 2min

94°C 30secs

63°C 1min   } 30 cycles

72°C 1 min

72°C 7 min

Dr Marcus Hughes designed the primer pairs and the sequences were as follows:

The product was resolved on a 1% agarose gel containing 0.1% EtBr, that was run in a

Bio-Rad sub-cell rig using 1x TAE (50x stock: 121.5g Tris, 9.3g EDTA, 28.55ml glacial

acetic acid) also containing 0.1% EtBr at 80V for 2 hours. Samples were loaded using 1x

loading buffer supplied with the kit. Gels were viewed using a UV-transilluminator and

photographed using a polaroid camera.

2.10.3 Ribonuclease Protection Assay

This method employs the use of endonucleases to digest single stranded but not double

stranded nucleic acids. A DNA probe complementary to the *EGFR* mRNA was kindly

prepared from cloned DNA by PCR by Dr Marcus Hughes. All other reagents were

supplied in the RiboQuant kit (Pharminagen, USA). The mRNA is extracted as in section

2.10.1 and then ethanol precipitated at -70°C for 1 hour before resuspension in the

hybridisation buffer supplied. The probe was radiolabeled using [α-^{32}P] ATP and the *in-vitro* transcription kit as stated in the manufacturers instructions (Pharminagen, U.K.) before mixing with mRNA overnight at a ramped temperature from 90°C to 56°C. The following day any unhybridised RNA was digested using endonucleases leaving only the hybrid RNA and probe protected from endonuclease digestion. The radiolabeled RNA: DNA duplex was separated by denaturing gel electrophoresis on a 6% urea polyacrylamide gel (12ml 19:1 30% acrylamide, 6ml 10x TBE, 27ml dH$_2$O, 25.1g urea, 600μls 10% AMPS, 40μls TEMED) and visualised by autoradiography and analysed by autoradiography as in sections 2.4.1-2.4.2 respectively.

2.11 Apoptosis Studies

To determine the method of cell death shown by treatment with ODNs the method employed looked at the DNA fragmentation found in the nucleus of cells upon apoptosis.

2.11.1 Fluorescence-activated Cell Sorter

Fluorescent-activated cell sorting (FACS) is a method based on flow cytometry that allows fluorescently labelled cells to be sorted according to the fluorescence they emit as they flow through a laser beam.

2.11.1.1 Cell Preparation

Cells were plated in complete media onto 24 well plates at a seeding density of 5×10^4 cells / well in triplicate for each ODN dose used. Cells were left to settle for 24 hours and then incubated in serum-free media with the appropriate ODN and lipid required for 4 hours and then fresh media added for a further 20 hours. After treatment cells were trypsinised using $2 \times 150\mu l$, counted and pelleted as in section 2.6.3 and then resuspended in a labelling mixture (0.1% triton X100, 0.1% sodium citrate, 0.5mg/ml propidium iodide) overnight at 4°C in the dark. The media was also collected containing the dead cells and pooled together with the trypsinised cells. The mixture enabled the cells to be lysed allowing the propidium iodide (PI) to intercalate with the DNA at a constant ratio. Thus the amount of PI is directly proportional to the amount of DNA present.

2.11.1.2 FACS Analysis

Cells were sorted for their DNA content using a standard FACS protocol for cell cycle DNA distribution analysis on a coulter counter (Coulter Corp., Fl. USA). A minimum number of 10000 events were counted at a medium flow rate and all experiments were repeated in triplicate over various passages of cells.

2.12 Statistical Analysis

Results were subjected to statistical analysis by the students unpaired t-test assuming equal variance (Gaussian population) using the Microsoft excel statistical software package. Significant differences between results were assumed when the two-sided P

values were calculated below 0.05. Unpaired t-tests assumed that data were randomly sampled, that each value was obtained independently of the others and that the populations were scattered according to the Gaussian distribution.

Chapter 3.0: Design of ODNs using DNA chip technology and optimisation of delivery and uptake in A431 cells

Since the discovery of antisense oligonucleotides as a potential therapeutic agent significant progress has been made concerning the stability and delivery of these drugs. However, perhaps one of the most difficult areas to be overcome in the potential of using these drugs effectively is the design of the specific antisense sequence to the target. In this study we have used a relatively new technique, DNA chip technology, to help design optimally acting ODNs for antisense work. This technique has been used here to predict accessible sites along the first 120 bases of the *EGFR* mRNA structure and to design antisense ODNs to these sites. Furthermore, we have used this technology to design a novel control sequence that is predicted to be unable to bind to the mRNA due to lack of accessibility. Once designed these sequences have been investigated for stability and modified forms of the ODN made to confer increased stability both in serum and serum-free media. Finally the delivery of these ODNs in A431 cells has been optimised using lipofectin^{TM} reagent and the cell growth assays required to determine the efficacy of the compounds *in vitro* developed.

3.1 Design of Sequences by Array Chip Technology

One of the initial hurdles to overcome when investigating antisense knockdown of a gene is the selection and design of the ODN sequence. It was widely thought that ODNs targeted to the start initiation codon area of the mRNA sequence would be a feasible target for ODNs to bind to. Studies initially targeted the initiation codon as it was thought that this region would be most accessible to the ODN (for review see Lebedeva & Stein, 2001). However, after many unsuccessful attempts to show sequence-specific knockdown of the target (Coulson *et al.*, 1996; Storey *et al.*, 1991) by this technique the problem of antisense design became apparent. A sequence walking technique has since been utilised which selects various sequences along the length of the mRNA and the full library of ODNs chosen can then be tested *in vitro*. Although this technique has identified some ODNs that are significantly biologically active the success rate is poor. Monia *et al.*, (1996) showed that only one ODN from a panel of 34 sequences was capable of decreasing protein and mRNA levels of *CRAF-1*, whereas Peyman *et al.*, (1995) showed that only one ODN targeted to the HIV-1 virus reduced the cytopathic effects from a panel of 100 sequences identified. It is clear that this technique is both time consuming and expensive with little guaranteed success. The use of computer folding programs that predict the secondary folded structure of the mRNA molecule in order to ascertain the most accessible sites have also been used in the design of antisense sequences. These programs however have now been shown to be ineffective in the prediction of potential targets with contradictory results found. Lima *et al.*, (1997) showed that the single stranded structures on hairpin loops were good sites whereas Laptev *et al.*, (1994) showed that the most potent antisense sequences were those targeted to double stranded regions of the mRNA. The lack of knowledge of factors that govern heteroduplex formation and

the secondary structure of the mRNA have shown the need for empirical screening methods to predict accessible sites for high affinity binding of an antisense ODN. Although pre-screening methods in cellular extracts is a good theory they are not practical for large scale testing of sequences required to find the most potently active ODN.

The development of DNA chip technology has provided a simple tool that allows screening of a vast number of sequences binding ability to their target. So far scanning arrays have shown a good correlation between binding strengths and antisense activity in a number of screens (Milner *et al.*, 1997; Sohail & Southern, 2001) however to date there has been no published work involving scanning array predicted antisense ODNs in mammalian systems.

Work was performed by the Department of Biochemistry, Oxford to predict antisense sequences that would bind to the first 120 bases of the human *EGFR* sequence. It was hoped that the predicted sequences could be synthesised and tested *in vitro* for their efficacy on *EGFR* in a model cell line that over-expresses the target gene. Figure 3.1 shows the predicted secondary structure of the first 120 bases of the *EGFR* mRNA by computer M fold programming and is used as a comparison of the type of sequences that could be determined using this method *versus* those selected by DNA chip technology.
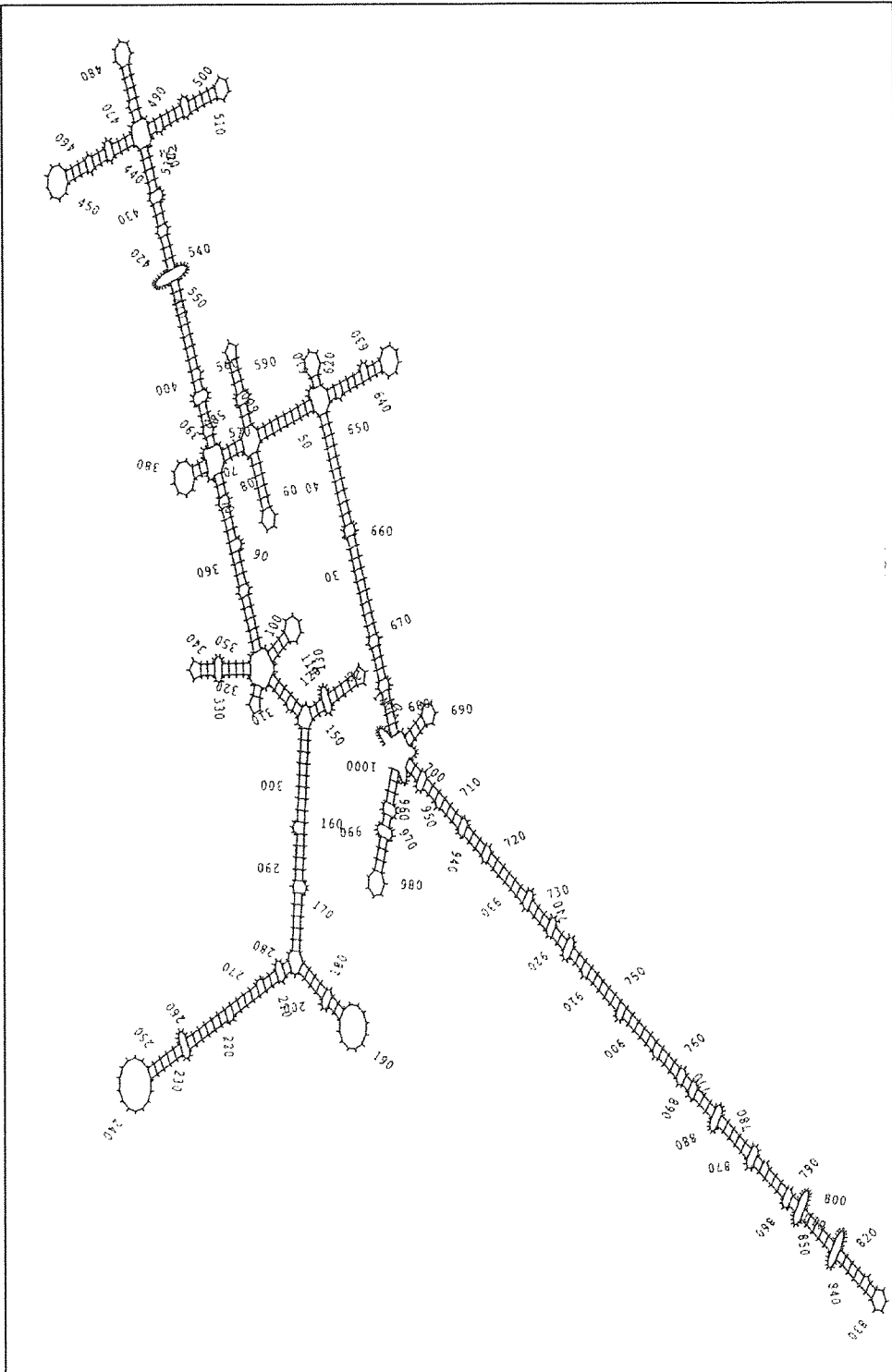
Figure 3.1: MFOLD of linear *EGFR* mRNA from bases 1 to 1000.
Predicted secondary structure of the *EGFR* human mRNA from bases 1 to 1000 by MFOLD programme at an annealing temperature of 37°C.

Interpretation of this data would normally suggest that sequences within single stranded regions such as hairpin loops or long stretches of linear areas would be the most accessible to binding by antisense ODNs. Upon performing DNA chip technology, utilising the same sequence of the *EGFR* as above, the following surprising results were obtained (figure 3.2).
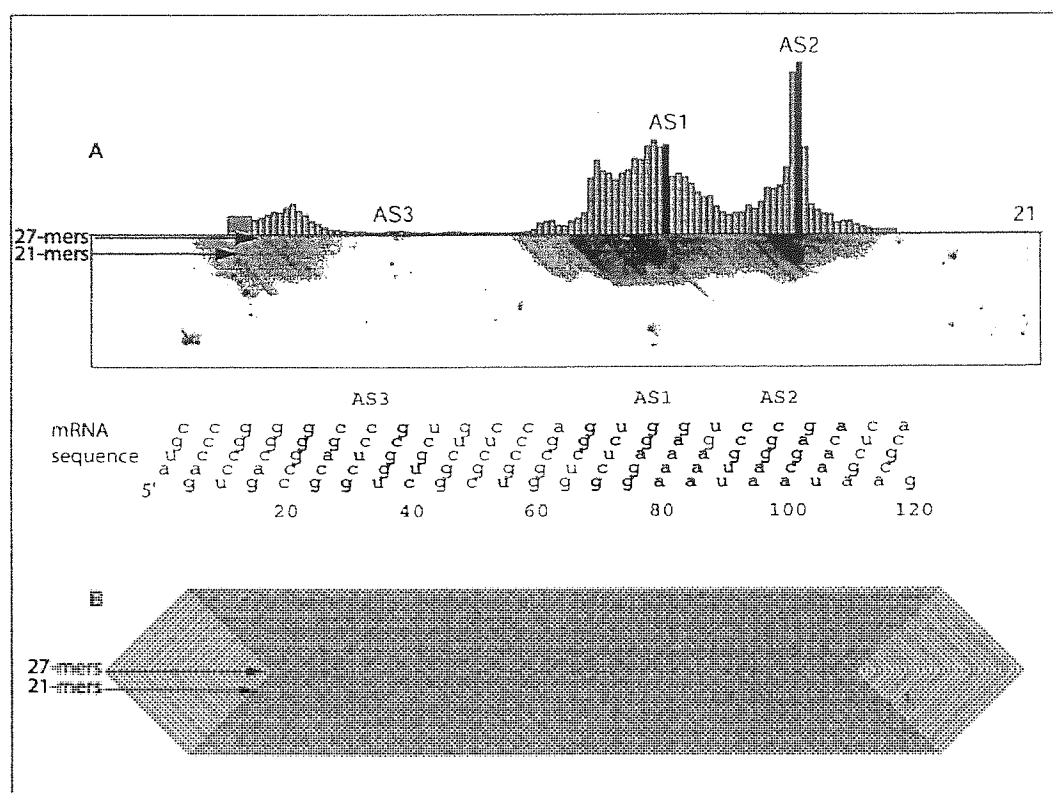
Figure 3.2: DNA chip technology display and analysis of results using xvseq:
A) Histogram of the hybridisation intensities for the 21-mer ODN along with an image obtained from ^{32}P-labelled mRNA to an array of complementary ODNs of all lengths and sequences up to 27-mer. Also displayed the mRNA sequence for the first 120 bases of the *EGFR* mRNA used (bold bases are those sequences selected). B) A template of the overlapping diamonds, corresponding in number to the sequence length which is generated and overlapped onto the hybridisation image to register the position and sequence of the ODNs in the array.

Interpretation of the array patterns obtained is aided by computer programming that enables a template grid of the layout of the ODNs to be overlaid onto the hybridisation pattern obtained with the radiolabeled mRNA. The strong signals from the radiolabel indicate the mRNA has bound to the ODN sequence at that particular site and thus hybridisation is not only a possibility but also the interaction is strong. Many sequences

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can be predicted of any length depending on which ones show the greater binding. The 21–mer length ODN not only showed good binding but is also of a length where upon no other sequence along the entire mRNA is likely to or does match. The problem with shorter ODNs, although they will hybridise quite well is that they are likely to occur again at some point on the sequence and thus the longer the ODN the more likely the sequence is unique. Alternatively, longer sequences will show good sequence specificity but are likely to be more difficult to deliver and also less likely to hybridise completely. From the array pattern two sequences were found to bind effectively and were termed AS1 (bases 67-87) and AS2 (bases 89-109). A third sequence was termed AS3 (bases 23-43) that did not show any binding efficiency and was thus used as a control. Although sequences close to that chosen for AS1 also showed good hybridisation, AS1 was chosen due to the nature of the sequence in that it did not contain any of the currently known motifs found to give non-specific binding (Matveeva *et al.*, 2000). Other controls used were variations on the AS1 and AS2 sequences and included scrambled and mismatch sequences. In order to confirm the length of ODN that was likely to be the most effective, various lengths of AS1 and AS2 were tested. All of these ODNs are summarised in table 3.1 below. The DNA chip technology has predicted antisense sequences that may be active but surprisingly they are found in computer folding program prediction areas that would not normally be predicted as available sites. If these ODN sequences are found to be effective the technique will also prove that current methods of computer prediction for mRNA structures are unreliable.

Sequence Name	SEQUENCE (5' – 3')
AS1 21mer	TTT CTT TTC CTC CAG AGC CCG
AS1 17mer	TT TTC CTC CAG AGC CCG
AS1 14mer	TC CTC CAG AGC CCG
AS1 10mer	C CAG AGC CCG
AS1 7mer	G AGC CCG
AS2	TGT TAC TCG TGC CTT GGC AAA
AS1 scrambled	CTG ATC CTG CTC TGA TCC TCT
AS2 scrambled	ATC GTC TTC AGT GAC TTA GCG
AS1 4 mismatch (mismatch bases in **bold**)	TTT CTT TTC C**GA TCG** AGC CCG
AS1 2 mismatch (mismatch bases in **bold**)	TTT CTT TTC CT**A TAG** AGC CCG
AS2 4 mismatch (mismatch bases in **bold**)	TGT TAC TCG **TTA ACT** GGC AAA
AS2 2 mismatch (mismatch bases in **bold**)	TGT TAC TCG TG**A ATT** GGC AAA
AS3	GCA GCG CCA GGA GCG CTG CCC

Table 3.1 Summary of all ODN sequences used and designed with the use of DNA chip technology against the first 120bp of the *EGFR* mRNA and subsequently synthesised for use in efficacy studies.

3.2 Stability in Biological Milieu of ODNs

Much of the initial work on stability of ODNs has shown us that unmodified DNA or RNA is very susceptible to degradation by endonucleases within the cell and its external environment. In order for a biological agent, such as an antisense drug, to be useful, it must remain intact long enough to reach its target of interaction and to initiate its effect. It was therefore important to address the issue of modification of the ODN sequences selected by DNA chip technology. Phosphorothioate modifications are still among the most widely used of the ODN modifications and are the most widely reported (Monia *et al.*, 1996; Roh *et al.*, 2000; Tian *et al.*, 2000). They have the advantage of being extremely stable as well as the ability to activate RNase H degradation of the mRNA target. The main disadvantage reported with this modification is its non-specific attraction to proteins within the cell and thus often produces false positive results of antisense effect (Burgess *et al.*, 1995; Barton & Lemoine, 1995). This issue was

addressed with the rigorous use of controls in the hope to prove that even with a renowned non-specific ODN modification, DNA chip technology can predict sequence-specific ODN sequences that can be used in antisense experiments. In order to clarify the stability of the phosphorothioate modification over the normal phosphodiester DNA structure, stability studies were performed in both complete media and in serum-free media. Further comparisons to a 2' O-methyl derivative, a stable RNA modification were also made.

3.2.1 Stability of AS1 in Complete Serum Containing Media

AS1 was radiolabeled as described in section 2.3 and assayed for stability in serum containing media over time (section 2.5). The AS1 sequence was synthesised as phosphodiester (PO), phosphorothioate (PS) and 2' O-methyl (2'O-me) derivatives to compare the relative stabilities and half lives of each. Serum displays significant nuclease activity and serum stability is frequently used in the antisense field as a general indication of the extra-cellular stability of molecules in vivo. Although the nuclease activity of sera derived from different species varies, foetal bovine serum (FBS) is reportedly more active than either mouse serum or indeed human serum (Crooke, 1992) and thus provides an excellent model for future stability.

Time (hours) 0 0.25 0.5 0.75   0 0.25 0.5 0.75 1 2 4 6 8 12 24 48

0 0.25 0.5 0.75 1 2 4 6 8 12 24 48   Time (hours)
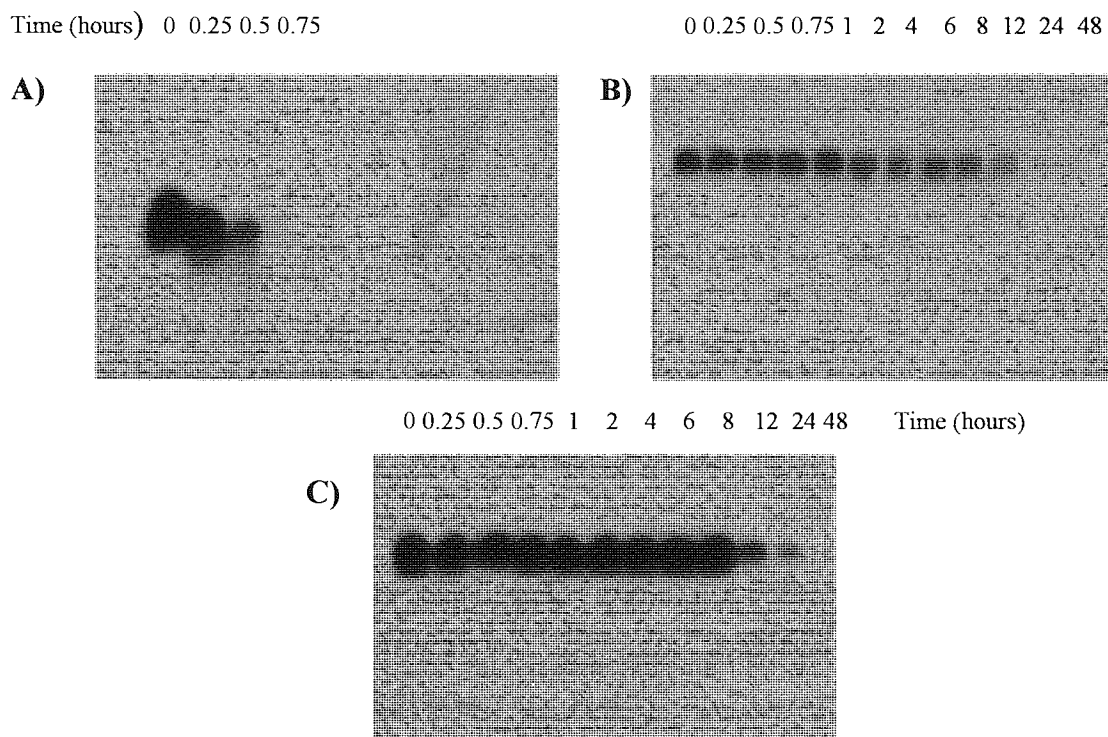
Figure 3.3: Stability of 5'-end ^{32}P-labelled 21mer AS1 in serum containing media over time.
Degradation profiles of **A)** PO AS1; **B)** PS AS1 and **C)** 2' *O*-methyl AS1 over time in complete media containing 10% FBS. ODN was added to 300μls of DMEM media and incubated at 37°C over the times indicated. 0 hours represents unexposed ODN. Degradation profiles were analysed by 20% 7M urea PAGE (section 2.5). Results are representative of triplicate experiments.

Phosphorothioate and 2' *O*-methyl chemical modifications applied to the ODN provided substantial protection from the nucleases present within the FBS / media compared to the profile obtained with unmodified PO ODN. The PO ODN was completely degraded within 45 minutes of addition to the media compared to the modified ODNs, PS and 2'*O*-methyl, which were stable for up to 12 hours and 24 hours respectively. Graphical representations of the degradation profiles as analysed by densitometry are shown in figure 3.4.
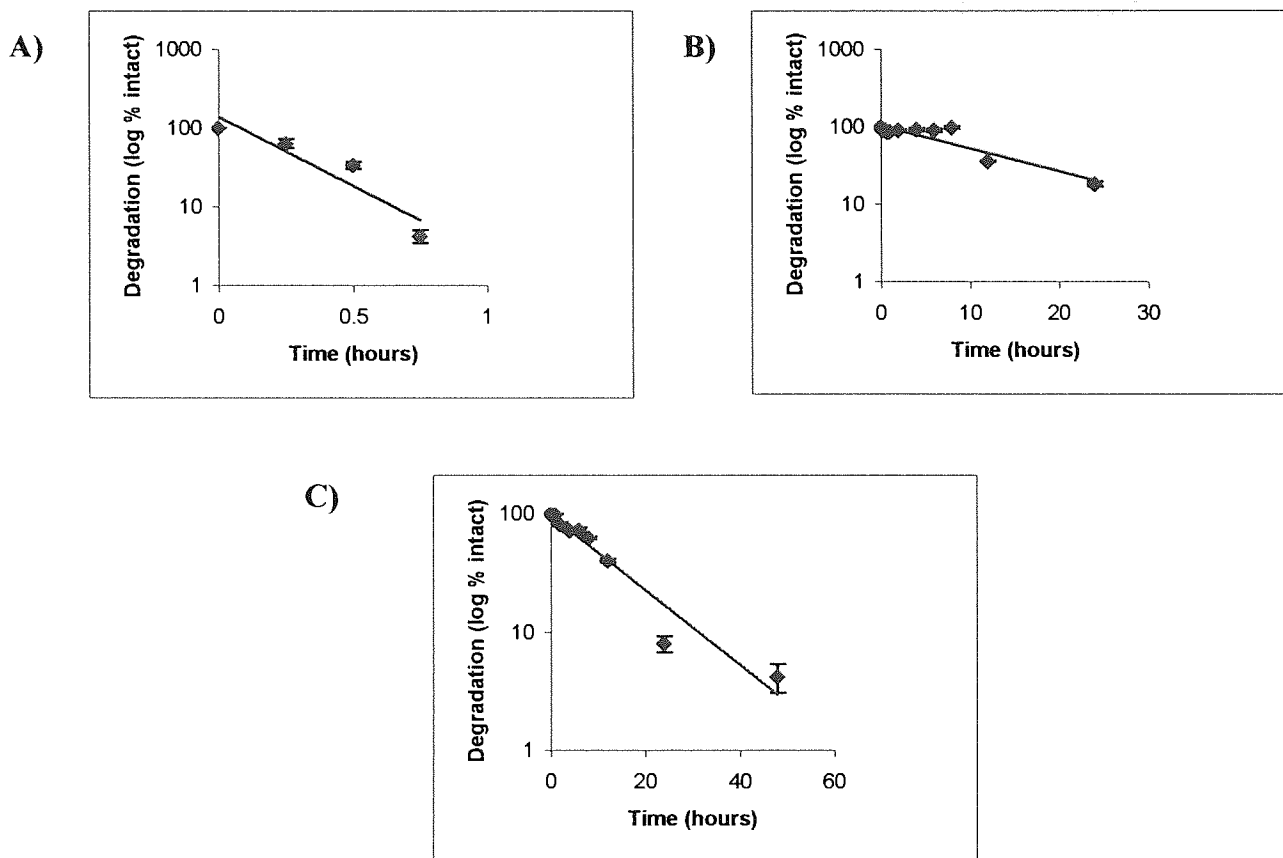
Figure 3.4:Graphical representations of stabilities of 21mer AS1 ODN in complete media over time.
Degradation profiles of A) PO AS1; B) PS AS1 and C) 2' *O*-methyl AS1 over time in complete media containing 10% FBS. Gels from figure 3.3 were analysed by densitometry and expressed as log of the percentage intact ODN with time. Results are representative from triplicate experiments (n=3 mean±SD).

The half-lives of the ODNs in the presence of serum media were 15 minutes for the PO ODN, 9 hours for the 2' *O*-methyl ODN and 11 hours for the PS ODN. Thus the modifications used conferred greater stability than that found with the unmodified PO ODN, which underwent degradation in a matter of minutes with complete degradation occurring within 1 hour. Surprisingly the PS ODN showed a greater stability half-life than that of the 2' *O*-methyl ODN but the latter modification still showed some ODN present after 48 hours compared to 24 hours with the PS ODN. PS ODNs have been shown to be up to 100 times more stable in serum than PO ODNs (Akhtar *et al.*, 1991;

Wagner *et al.*, 1993) and in this case although they were more stable the increase was approximately 44 fold higher. 2' *O*-methyl ODNs have been shown to give equal or greater stability than PS ODNS (Brown-Driver *et al.*, 1999;) and in this case only showed similar stabilities to the PS ODN. Importantly though the 2' O-methyl derivative still showed increased stability over PO ODNs with a 36-fold increase in stability over the PO ODN. Although the results obtained do not agree completely with those stated in the literature it is acknowledged however, that the nuclease activity in FBS varies considerably between batches and it is therefore difficult to make direct comparisons with other studies.

3.2.2 Stability of AS1 in Serum-free Media

As the FBS within the media is the main source of nucleases responsible for the degradation of ODNs *in vitro* it is often found that ODNs are administered in the absence of serum. Furthermore delivery methods employed for ODNs are often inactivated by serum and thus the environment has to be serum-free to ensure a greater possibility of the ODN entering the cell and reaching its target. The delivery method employed in this study uses cationic lipid delivery and requires the use of serum–free media, thus the stability of ODNs in serum-free media also needs to be investigated. Although delivery methods and modifications have been developed that are active and stable in serum media, this study is more concerned with the efficacy of designed ODNs. As such the methods employed here are more commonly used delivery methods and modifications so that a comparison can be more easily made with current literature on the efficacy of the ODN.
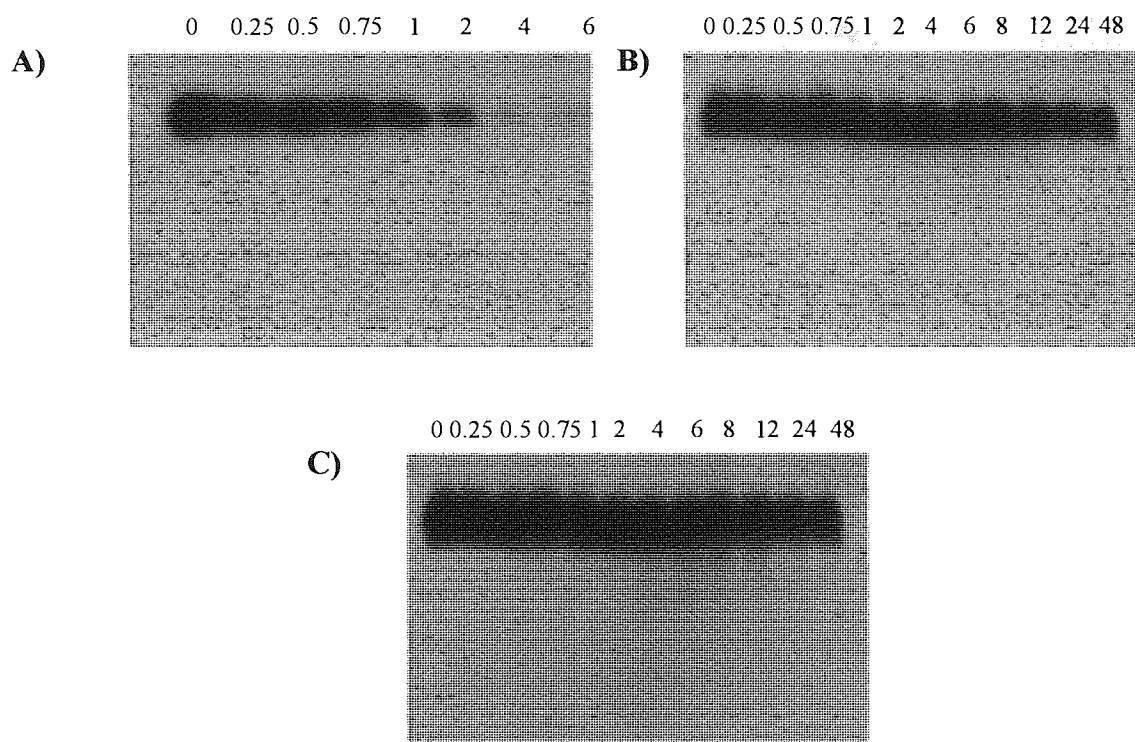
Figure 3.5: Stability of 5'-end ^{32}P-labelled AS1 in serum-free containing media over time.
Degradation profiles of A) PO AS1; B) PS AS1 and C) 2' *O*-methyl AS1 over time in serum-free media containing 0% FBS. ODN was added to 300μls of DMEM media and incubated at 37°C over the times indicated. 0 hours represents unexposed ODN. Degradation profiles were analysed by 20% 7M urea PAGE (section 2.5). Results are representative of triplicate experiments.

The chemical modifications applied to the ODN showed similar degradation profiles as those found within the serum containing media experiments with the degradation of the PO still occurring much more quickly than that seen with the PS and 2' *O*-methyl ODNs. The PO ODN was completely degraded within 8 hours whereas the modified ODNS were still very much present after 48 hours in serum-free media. Graphical representations of the degradation profiles as analysed by densitometry are shown in figure 3.6.
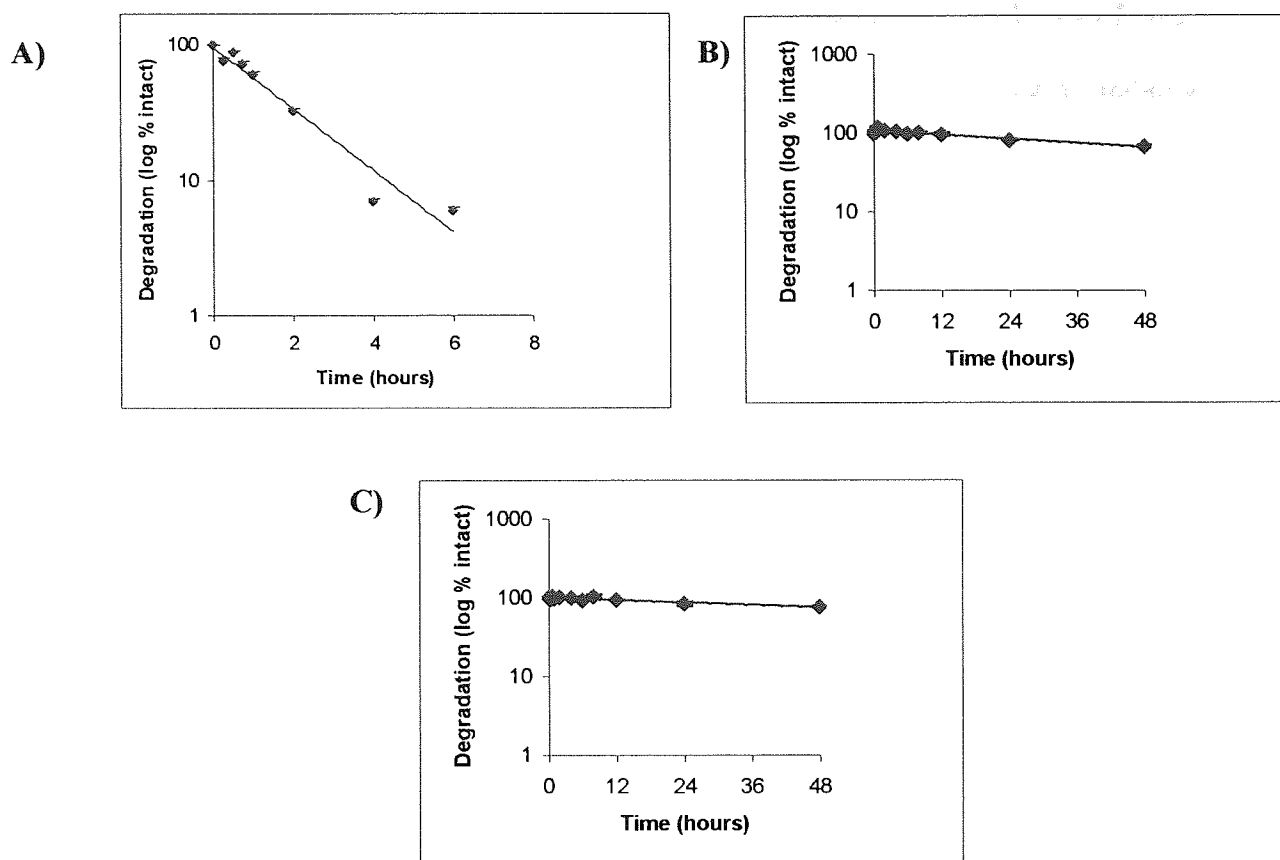
Figure 3.6: Graphical representations of stabilities of AS1 ODN in serum-free media over time.
Degradation profiles of A) PO AS1; B) PS AS1 and C) 2' O-methyl AS1 over time in serum-free media containing 0% FBS. Gels from figure 3.5 were analysed by densitometry and expressed as log of the percentage intact ODN with time. Results are representative of triplicate experiments (n=3 mean±SD).

The half-lives of the ODNs in serum-free media were 1.25 hours for the PO ODN, 75 hours for the PS ODN and 118 hours for the 2' O-methyl ODN. All of the ODNs showed greater stability in serum-free media than in serum containing media as serum-free media contains much less nucleases thus degradation of the ODNs is less likely. The modifications used conferred greater stability than that found with the unmodified PO ODN, which underwent degradation within a little more than an hour with complete degradation occurring within 8 hours. As expected the PS ODN underwent only slight degradation over the 48 hours period as well as the 2' O-methyl degradation. In this case the half-lives agree with the literature in that the 2' O-methyl ODN showed less

degradation over time than the PS ODN and thus confers greater stability (Brown-Driver *et al.*, 1999, Coulis *et al.*, 2000). The PS ODN showed a 60 fold increase in stability compared to the PO ODN and the 2' *O*-methyl ODN showed a 94 fold increase in stability over the PO ODN. The 2' *O*-methyl ODN gave a 1.5 fold increase in stability over the PS ODN.

These results show that using the modifications of PS and 2' *O*-methyl gives a greater stability of the ODNs both in serum media and in serum-free media thus providing a greater scope for use *in vitro* and *in vivo*. These modifications are some of the more widely used with the PS ODN having the advantage of activating RNase H degradation of the target mRNA. There are many modifications now available that allow greater stability compared to the above modifications as well as less side effects such as the non-specific effects found with the PS ODN. Such modifications now include peptide nucleic acids (Hamilton *et al.*, 1999; Shammas *et al.*, 1999), 2'-methoxyethyl- (Khatsenko *et al.*, 2000) and morpholino-based ODNs (Arora *et al.*, 2000; Qin *et al.*, 2000). However, this thesis is more concerned with the efficacy of the ODNs designed by the DNA chip technology and their potential use in the clinic. Although as a therapy it is important to address the aspects of delivery and stability this is likely to be dependant on the target employed and the therapy regime. For the purpose of testing efficacy of the ODNs it is acceptable for the ODNs to be stable over the treatment period and for them to be deliverable to their target.

3.3 Cellular Association of ODN in A431 Cells

As a result of the promising stability results found with the modified ODNs the issue of delivery of the ODNs into the cells was addressed. To enable the use of ODNs as a

laboratory tool and as a therapeutic agent it is important to ensure the optimal conditions are found for the delivery of the ODN to the cell. It is important that the maximum amount of ODN can be delivered to the cell and also that the ODN is reaching its target before degradation. In the efficacy studies following, the A431 cell line is used as a model cell line for the study of the ODNs as it over-expresses *EGFR* (Ullrich *et al.*, 1984), the target for which the ODNs are designed. Due to this the association studies employed were performed on this cell line. However, if the ODNs are to be used in the clinic it is postulated that the delivery procedure will need to optimised further dependant on cell type.

It is possible to use a variety of delivery methods for ODN delivery to the cell (section 1.3) and as previously stated the aim of this study is not to do an exhaustive study of the various methods available. The method employed was that of cationic lipid conjugation. The lipid employed was a commercially available mix of N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA) and dioleoyl phosphatidylethanolamine (DOPE), which is susceptible to inactivation in serum conditions, thus needs to be administered in serum-free conditions. The fusogenic DOPE acts as a helper lipid to destabilise the endosomal membrane, thus permitting release of nucleic acids from endosomes once inside the cell (Jaaskelainen *et al.*, 1998). A recent report shows that the efficiency of ODN uptake is dependant on the ratio amounts of ODN to lipid and this can differ between cell types and thus needs to be optimised in each case studied (Islam *et al.*, 2000; Williams & Buzby, 2000). Due to this cellular association studies were employed to determine the optimal ratio of ODN to lipid to use.

A431 cells were plated at a concentration that would ensure they were in an exponential phase over the period of time used. This had previously been determined in the laboratory to be at 1×10^4 cells/ml in a 24 well plate (Fell, 1997). A431 cells were plated out in

serum-free medium at a concentration of $1x10^4$ cells/ml and incubated for 4 hours at 37°C with various ODN: lipid concentrations ranging from 1:0 to 1:5. The ODN was 5' ^{32}P-radiolabeled and complexed with lipid prior to adding to the cells for 4 hours. After 4 hours it is documented that the maximum uptake of ODN into cells will have occurred (Yakubov *et al.*, 1989; Levis *et al.*, 1995) thus this was deemed sufficient time to study the association of ODN with the cell. Following 4 hours the cells were washed to remove any loosely associated ODN complex so that false results of associated ODN with the cell are not obtained. In order to determine the number of washes required for the removal of loosely associated ODN a procedure was performed where upon the ODN alone was added for 4 hours and then washed up to 6 times with each wash collected separately and analysed for radioactive content. It was deemed that all of the loosely associated ODN was removed when minimal radioactivity was recorded in the wash sample. Furthermore, due to the stringency of the wash, cell viability was also determined by visual comparison to control cells, as removal and damage to cells would release internalised ODN and give increased radioactivity and thus false results. Cells were intact and attached to the plate following each wash.
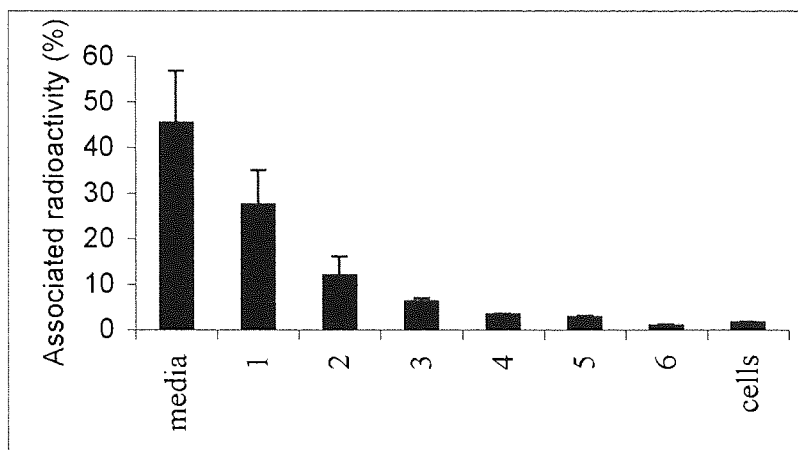
Figure 3.7: Determination of number of PBS-azide washes required to remove all loosely associated radioactivity.
Following incubation with radiolabeled ODN the apical media was collected and the cells washed with 0.5ml of PBS-azide for 5 minutes. Each wash was collected and after 6 washes the cells were lysed with 0.1% triton-100 and collected. All samples were analysed for radioactive content by LSC (section 2.4.3). The amount of unbound / loosely bound ODN associated with each fraction (i.e. media, respective washes, cells) is expressed as mean (n=3 ±SD) % of total ribozyme associated radioactivity applied to the monolayer.

It is evident that from the wash results one or two washes is insufficient to remove the loosely bound ODN and that after 6 washes the majority of the ODN is removed. Observing the cells at this stage prior to lysis showed that they were still attached to the plate and intact thus the washes were not damaging to the cells. From this it was decided to use 6 times 0.5ml washes for 5 minutes *per* wash in all subsequent association experiments.

As stated above the ratio of positive to negative charge is known to be an important factor in transfection, thus studies were undertaken to determine the optimal charge ratio for the lipid formulation, lipofectin[TM], required to increase cellular association in A431 cells. A neutral to positive charge is best for transfection (Hope *et al.*, 1998; Jaaskelainen *et al.*, 1998) and thus the ODN concentration was kept constant and the amount of positively charged lipid increased to provide a greater positively charged complex.
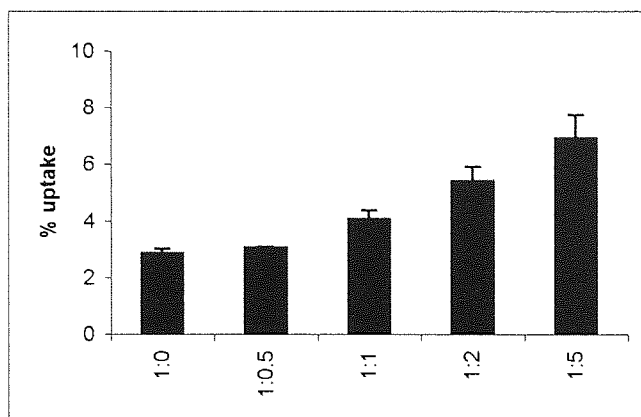
Figure 3.8:Optimisation of the ratio of ODN: lipid required for the greatest association of ODN with the cell.
Cell association of 0.5µM ODN when complexed with lipofectin^{TM} at various charge ratios after 4 hours incubation at 37°C was determined. Cells were washed six times as summarised in figure 3.7 before analysis of cell lysate for radioactivity and thus calculation of asociated ODN with the cell. % uptake was determined by LSC and expressed as results from triplicate experiments (n=3 mean±SD).

From the above results it can be shown that the greater the amount of lipid present the greater the amount of association of the ODN with the A431 cells. Thus the greater the positive charge the greater the cellular association. Thus from these results it can be concluded that the best ratio to use of those tested would be the 1:5 ODN:lipid ratio. Although it is has been shown here that the uptake of ODN using cationic lipids is relatively rapid and dependant on ratio it remains to be seen whether the process is temperature dependant. All of the results concerning association so far have been performed at 37°C but it is important to determine the effect of temperature on the process as this enables us to determine whether the process is an active energy dependant process.

Cells were incubated with 500nM AS1 ODN for 4 hours at either 4°C or 37°C prior to washing and collection of lysed cells for analysis of radioactive content.

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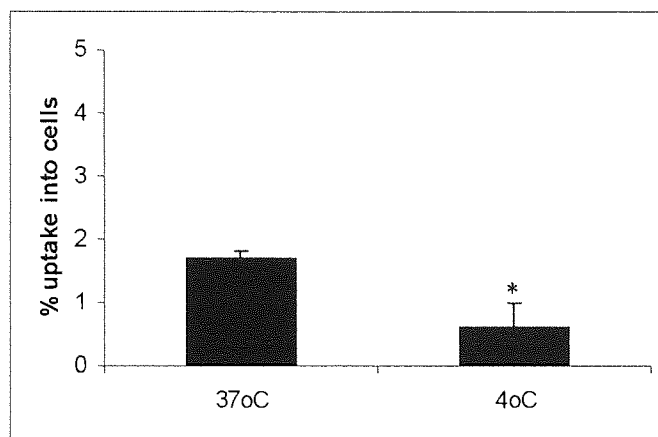
Figure 3.9: Association of ODN with A431 cells at various temperatures.
Cells were assessed for ODN association at 4°C and 37°C over a 4-hour period by incubation with 500nM ODN in serum media. Cell association is expressed as a total percentage of the initial amount of radiolabel added to the cells. Results represent triplicate experiments (n=3 mean ±SD). * indicates significant difference (p<0.05) from 37°C.

The results show that the uptake of ODN is significantly greater (p<0.05) at 37°C than at 4°C indicating that the process of ODN uptake is temperature dependent. The requirement of heat for the process indicates that the process is an energy dependant process and is concurrent with the literature (Yakubov *et al.*, 1989; Loke *et al.*, 1989). An energy dependant process indicates that the association of ODN with the cell is by an endocytic or other energy dependant-type mechanism and discounts the possibility of a passive-type mechanism.

3.4 Optimisation of Cell Number Determination Assays

In an ideal situation it would be feasible to use the maximum concentration of lipid to ODN possible to ensure enhanced ODN association with the cell. However, it is widely reported that high levels of positively charged molecules such as lipids can also be toxic to the cell (Filion & Phillips, 1997), with different cell types having different threshold

levels to different lipids (Miyake *et al.*, 1999; Gleave *et al.*, 1999). Due to this fact a preliminary study was performed to determine the toxicity of the lipofectin[TM] reagent at various concentrations to the cells. Cell toxicity studies were performed in 96 well plates and assayed for cell number and viability by crystal violet assay. In order to determine the cell number after each treatment a standard curve needed to be constructed to ensure the OD values obtained for the samples could be compared to cell number in samples.

The figure shows a plot with OD 550nm on the y-axis (0.0 to 3.5) and Cell number on the x-axis (0 to 100000). The equation displayed is:

$$Y = 3.116e^5 \times X + 0.255006$$
$$r^2 = 0.992$$
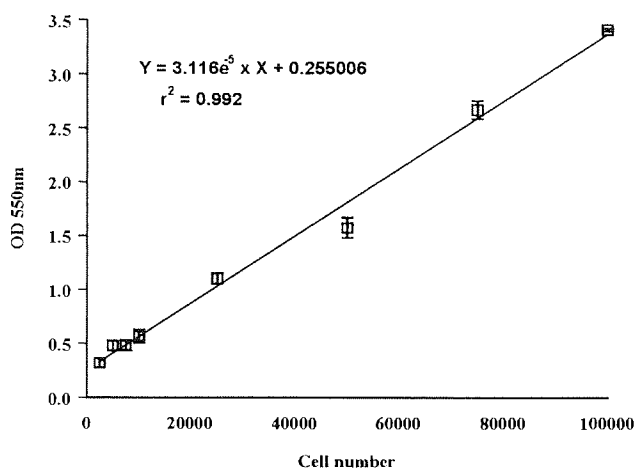
Figure 3.10: Representative standard curve forA431 cells assayed by the crystal violet assay. Cells were plated in 96 well plates at various concentrations and then assayed by crystal violet assay as described in section 2.8.1.2.

Cells were seeded in wells and incubated at 37°C for 4 hours. This enabled the cells to settle and attach to the plate but was not sufficient enough time for the cells to divide so that an accurate comparison of cell number versus optical density can be made.

Growth rates of A431 cells also had to be studied in order to determine which cell number would be best for the initial seeding density of treated cells so that the cells did not reach confluence before the assay was complete.
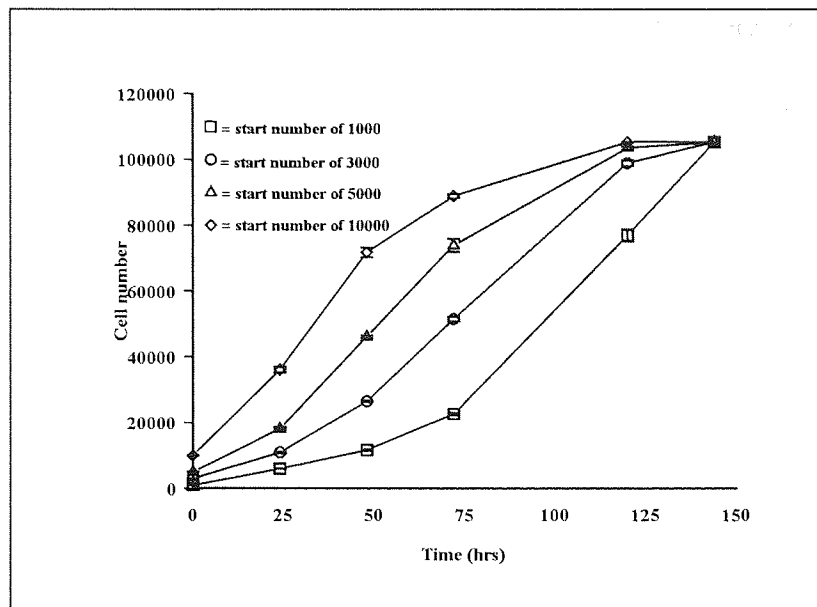
Figure 3.11: Growth curves of A431 cells over time when seeded at different initial densities. A431 cells were seeded in 96 well plates at initial starting densities of 1, 3, 5 and 10 X 10^3 cells/ ml and assayed for growth over time by the crystal violet assay (section 2.8.1.2). Error bars represent n=4 mean ±SD.

From these results it can be seen that all of the seeding densities chosen allowed the cells to grow, although each followed a slightly different pattern. The cells seeded at 1000 cells per well initially took a few days to grow at a linear rate and thus were in a lag phase until this point. It was not until 72 hours that the cells began to grow at a linear rate and thus were in an exponential phase of growth. When cells are present in a lag phase their growth characteristics will be different to cells in a linear growth pattern, in the respect that they will be in varying stages of the cell cycle. Some cells may be undergoing cellular division whilst others could still be in a stationary G0 phase. In order to compare the results of samples between populations from subsequent experiments the cells need to be in a linear phase and thus this seeding density was deemed inappropriate. On the other extreme for cells that were initially plated at 10000 cells per well, their initial growth rate for the first few days was linear and thus appropriate for cell studies. However at time points greater than 48-72 hours the cell growth rate steadies into a near stationary phase.

This phase indicates that although cells are still growing there are an equal number that are either not growing or dying. As transformed cells do not undergo normal contact inhibition and thus would be unlikely to stop growing due to lack of space (Freshney, 2000), it can only be postulated that the cells are dying, probably due to an increase in toxin levels or a decrease in nutrient levels. It is therefore summarised that this seeding density although suitable for time points up to 48 hours would not be suitable for experiments that required longer time points. Both the cell densities of 3000 and 5000 are appropriate for cell assays that require time points up to 120 hours as they both maintain a linear growth rate up to this point. After this the 5000 cell density does seem to flatten out into a stationary phase but the 3000 cell density is still in a growth phase and thus would still be suitable. The 3000 cell density does however have a small lag phase for the first 24 hours and thus would not be suitable for short time point studies on cell growth rates. As most of the cell efficacy work carried out is to be at times of 24-120 hours it was deemed that the 5000 cell density would be the most suitable for growth studies as the rate is almost totally linear throughout this period.

From these results it was summarised that for most studies the 5000 initial cell plating density would be used as it provided the best growth rate. This would provide cells in a constant proliferation state and thus any hindrance to cell growth observed could be attributed to treatment and not growth conditions.

3.5 Toxicity Studies of ODN and Lipofectin[TM]

As stated previously it would be ideal to use the maximum amount of lipid possible combined with the required dose of ODN to ensure that the association of ODN with the cell is maximal. However, there is the evident possibility that the ODN and the lipid

alone may be toxic to the cells at high concentrations. That is to say that a decrease in cell number would be observed following treatment with these compounds alone which would not be due to an antisense effect. Indeed ODNs that have been administered at high concentrations have been shown to be toxic to the cell as they bind to non-specific proteins and inhibit the cell in this way (Coulson *et al.*, 1996). In order to determine the toxicity of the ODNs and lipid alone they were administered to cells for 4 hours in the presence of serum-free media and then the cells fed with fresh media for a further 20 hours prior to cell number analysis by crystal violet assay.
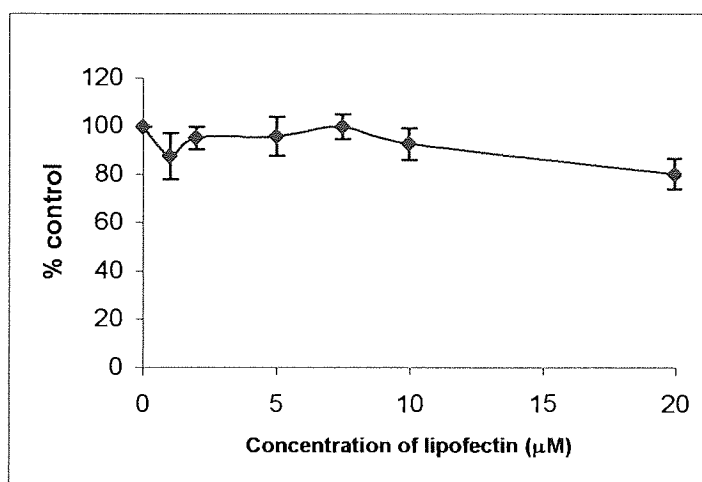
Figure 3.12: Effect of lipofectin concentration on cell number over 24 hours.
Lipofectin was added to cells at increasing concentrations for 4 hours in serum-free media and then fresh media added for a further 20 hours. Cell number was analysed by crystal violet assay (section 2.8.1.2). Error bars are representative of n=3 mean ±SD.

This result shows that the effect of lipofectin on cell number is relatively non-toxic up to the concentration of 10μM but above this the cell number starts to decrease showing that the lipid is toxic to the cells. As stated previously (section 3.3) the ratio of ODN to lipid is important and from the association studies in figure 3.8 the higher the ratio of lipid to ODN the better the association with the cells. However, these studies indicate that this may not be plausible as the toxicity of the lipid at these ratios may cause some effect. For

example the charge on a 21mer ODN is –20 and for each lipid molecule there is a +1 charge. Thus to give a 1:5, ODN: lipid charge ratio at an ODN concentration of 500nM a concentration of 50μM lipid would be required which would be extremely toxic to the cells. From these results it was decided that the maximum concentration of lipid to be used in subsequent assays would be 7.5μM to be sure that no toxicity would occur due to the lipid alone. Although studies have worked with ratios of ODN to lipid to ensure constant association of the ODN with the cell at different concentrations (Tian *et al.*, 2000; Coulis *et al.*, 2000) it is not plausible to do this in this case. Recent antisense ODN studies using lipofectin have now adopted the approach of a constant lipid concentration to ensure no effect of toxicity by the lipid and use a variation in the ODN concentration and thus overall ratio (Monia *et al.*, 1996; Roh *et al.*, 2000; Wu-Pong *et al.*, 1994). It was decided from these results here that future studies with the DNA chip designed ODNs should adopt this approach.

Similar experiments were carried out with the ODN alone in its phosphorothioate form (the main chemistry used throughout these studies). Many studies using PS ODNs use ODN concentrations in the nM and μM range (Coulson *et al.*, 1996; Coulis *et al.*, 2000; Tian *et al.*, 2000) thus a range of ODN concentrations between 10nM and 25μM were tested.
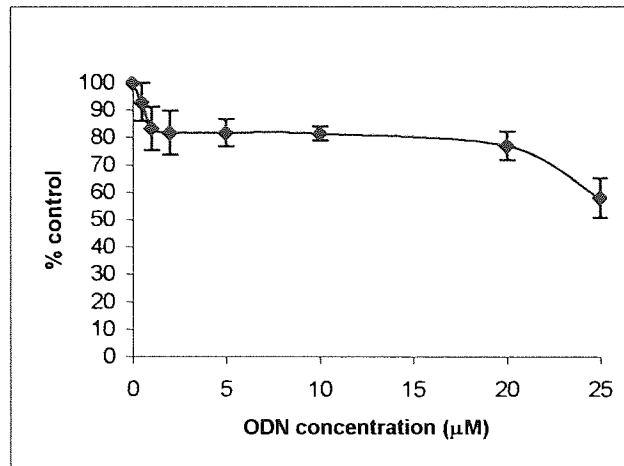
Figure 3.13: Effect of AS1 PS-ODN concentration on cell number.
The PS ODN, AS1 was added to the cells at various concentrations without any delivery agent. The ODN was administered to the cells for 4 hours followed by a further 20 hours incubation in fresh media before the cell number was assayed by crystal violet assay. Error bars are representative of n=3 mean ±SD.

The results for ODN toxicity show that the ODN even at very low concentration have a very small effect on cell number of about 20%. The initial decrease observed at even the lowest concentrations is indicative of the type of response seen by antisense molecules upon cells without a delivery system, in that a certain amount of the ODN will enter the cell and exert its effect (Bennett *et al.*, 1992). The results show that this effect seems to give a maximum inhibition of 20%, independent of increasing concentrations and this may be explained by the fact that receptors necessary to uptake the ODN are saturable at increased concentrations (Loke *et al.*, 1989). Following this initial effect the cell number does not decrease until the concentration reaches approximately 20µM at which point the cell number begins to decreases at increased concentrations. This sudden decrease is unlikely to be due to an antisense effect as no dose response was observed prior to this and is more likely to be due to a toxic non-specific side effect of the PS ODN. PS ODN are renowned for binding non-specifically to proteins and thus it can be postulated that at this higher concentration the cell surface proteins are being inhibited by the non-specific

binding of the ODN and thus essential receptor mechanisms of the cell that use these proteins are inhibited causing the cell to die. Other published PS antisense work carried out at high ODN concentrations shows that the ODNs are often working via a non-antisense effect (Coulson *et al.*, 1996).

3.6 Treatment Conditions

It has been shown that lipid complexes such as lipofectin are readily inactivated in serum conditions. Also ODNs, whether modified or native, degrade much more quickly in serum conditions compared to those of serum-free (section 3.2). Consequently A431 cells were treated with the lipid/ ODN complex in serum-free medium. As most ODN uptake has been shown to occur within 4 hours, cells were treated with the ODN / lipid complex in serum-free media for this time before being changed to fresh serum-containing media afterwards. This was the strategy that was first adopted in cell treatments but it was soon noticed that the cells once reintroduced into complete media were very unhealthy and began to die. Although this could have been due to the antisense ODN it was thought that not enough time had passed to enable the ODN to exert its effect.

A431 cells are shown to be able to survive in serum-free conditions due to their autocrine system of growth factor production (Tang *et al.*, 1997; Grandis *et al.*, 1998). The cells produce their own growth factors such as EGF and TGF-α, which are then released from the cell and bind to the cells own receptors, to stimulate further production of the factors and growth pathways such as the MAPK signal transduction pathway. It is due to this mechanism that tumour cells, such as gliomas, are thought to be able to survive in the body and enables them to provide increased nutrient concentrations for unlimited growth. In these studies it is also thought that this pathway could be the reason why the cells were

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showing early death. When the cells were re-introduced to serum containing media it is postulated that the concentration of growth factors was too great causing a toxic effect on the cells (Skarpen *et al.*, 1998).

In order to determine whether the cells could survive for longer periods in serum-free conditions a simple growth experiment was performed. The cells were plated in full media and left to grow for 24 hours after which time the cell number was analysed. A second batch of cells were then given fresh serum-free media for the following 24 hours and also assayed for further cell growth. It is postulated that if the doubling times of the cells are equivalent in both serum-containing and serum-free media then the cells are capable of surviving for at least 24 hours in serum-free conditions and this procedure can be adopted for future efficacy studies over this time frame.
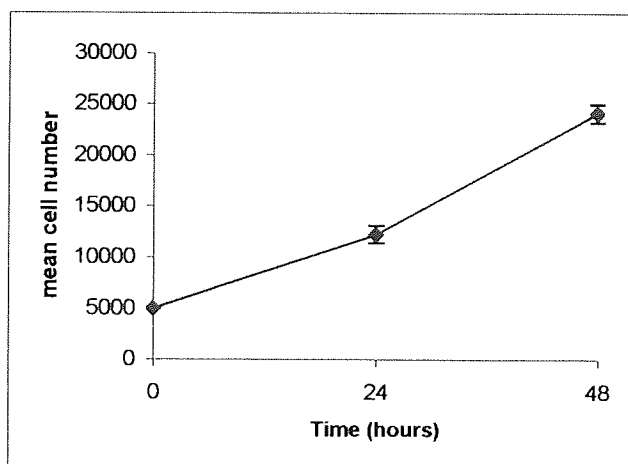
Figure 3.14: Growth of A431 cells in serum media followed by serum-free media over a total of 48 hours.
Cells were seeded in well plates in complete media with 10% FBS and left to grow for 24 hours prior to cell number determination by crystal violet assay. A second batch of cells was also treated as above but then left for a further 24 hours in serum-free media before being assayed in the same way. Error bars represent n = 3 mean ± SD.

The above results show that the A431 cells are still capable of growing for a further 24 hours in serum-free media after they have initially been seeded in serum-containing media. The doubling time for cells in the first 24 hours, i.e. those in complete media, is 19.6 hours where as the doubling time for the serum-free growing cells is 24.4 hours.

Thus although the cells are growing slightly slower in the presence of serum-free media they are still in a growing phase. This means that any effect observed on cell number with subsequent treatments will be due to the treatment and not the lack of nutrients in the media. This characteristic of cellular growth in the absence of growth factors can be attributed to the cells ability to grow in an autocrine manner when effectively starved of nutrients.

3.7 Conclusion

Secondary folding of an RNA molecule is a major determinant of its higher order conformation and its interaction with a variety of other molecules. As such the ability to design antisense sequences targeted to a particular RNA is dependant on knowledge of the structure of that RNA. This has been the main standing point to the design and prediction of antisense target sequences and the forward development of many antisense molecules in the clinic. As discussed there are many methods currently employed in the antisense field for the determination of antisense sequences but to date these are all usually inaccurate, expensive or often time-consuming. The use of DNA chip technology employed here has enabled us to predict two antisense sequences towards the *EGFR* mRNA that should be able to hybridise to the sequence they are directed at in such a way as to allow a specific knockdown of their mRNA target. In conjunction with this, the technique has allowed us to select a sequence that acts as a control both for antisense effect and for the selection procedure of the technology. The region of which the technology has predicted antisense sequences will bind is surprisingly located within unavailable motifs predicted by computer folding programs. If these ODN sequences are

found to be effective the technique will also prove that current methods of computer prediction for mRNA structures are unreliable.

In order to test the hypothesis that these ODNs are effective against their target it is necessary to ensure that the ODNs synthesised are stable in the biological environment. The two sequences chosen were synthesised as phosphorothioate and 2' O-methyl derivatives and were tested for stability both in serum -containing and serum-free media. Both modifications proved to give greater stability than the standard phosphodiester structure. Phosphodiester ODNs although in the past have shown antisense effect are now known to be rapidly degraded to monomeric nucleotides which in turn are toxic to the cell and are likely responsible for the assumed antisense effect observed (Vaerman *et al.*, 1997). The modified ODNs however were still almost completely degraded within 48 hours in the presence of serum but with the serum-free media were still very much intact after this time. This however does not pose a great problem, as the delivery agent employed in this study for delivery of the ODNs to the cell was that of lipofectin. Cationic lipids, although fairly stable, are not as efficient at forming complexes with the ODN in serum conditions and are thus more widely used in serum-free conditions. The amount of lipid required for the delivery of ODN to A431 cells also had to be optimised. It was found that the greater the overall positive charge of the complex, *e.g.* the greater the concentration of the lipid, the better the percentage association of ODN with the cell. However, further studies showed that at concentrations of lipid greater than 10μM the lipid was toxic to the cells such that a maximum dose of 7.5μM lipid was chosen for all subsequent experiments. Furthermore, ODN concentrations above 20μM were also found to be toxic to the cell when delivered alone thus this was the maximum concentration deliverable to the cell before non-sequence specific effects occurred. Consequently it was

concluded that a constant concentration of lipid would be used throughout as opposed to the traditional method of maintaining a constant ratio of ODN to lipid.

Finally as a result of these studies the effect of serum-free media on cell growth was determined. It was concluded that due to their apparent inherent autocrine growth characteristics, A431 cells were able to maintain a similar growth rate in serum-free media compared to that found in serum-containing media. Thus over a 24-hour period, efficacy studies could be performed in serum-free media without any detrimental effects to the cells growth characteristics other than those caused by the antisense molecule itself.

Chapter 4.0: Efficacy study of chip designed ODNs on A431 cells.

One of the main issues to be addressed when considering whether exogenously delivered ODNs, targeted to *EGFR*, provide a realistic therapeutic approach to the treatment of Glioblastoma Multiforme is whether ODNs will be effective in inhibiting the activity or expression of the chosen target protein. Thus as well as looking at the effect of the ODN on cell growth, it is also important to look at its efficacy on protein expression levels. Furthermore, if an effect is established it is useful to have an insight in to the mechanisms by which this effect is generated. This can be studied both by changes in the chemistry of the ODN and by examining the presence or absence of mRNA degradation. Finally if cell growth, protein levels and mRNA are inhibited, it is important to understand exactly what is happening to the cells to cause specifically any decrease in cell number. Is the mechanism of death due to toxicity or is it a knock on effect bought about by apoptosis due to the depletion of a vital growth factor, in this case EGF within the cell? All of these questions were addressed here as well as a thorough investigation of the type of controls that are necessary to establish that the effect observed is truly an antisense effect.

4.1 Effect on Cell Proliferation by Antisense ODNs and Control Sequences.

The research detailed in chapter three reveals that potentially effective ODN sequences identified by DNA-chip technology to target *EGFR* are few and thus are relatively quick and simple to test *in vitro* and *in vivo* for efficacy. Initials studies were carried out to determine whether ODNs delivered with optimised lipofectin^{TM} could inhibit cellular growth. As phosphorothioates proved to be stable in serum-free media for 24 hours (chapter 3) and are known to utilise RNase H cleavage pathways in their method of antisense activity, these were synthesised at various lengths between 7 and 21 bases (table 3.1). Studies have successfully demonstrated that efficacy with ODNs can be found at nanomolar concentrations (Monia *et al.*, 1996; Wagner *et al.*, 1993) thus a range of doses from 20nM to 1μM were screened for inhibition of cellular proliferation of A431 cells. ODN doses within the micromolar range have been used in many studies (Coulson *et al.*, 1996; Veal *et al.*, 1998; Roh *et al.*, 2000) however, it has been shown that in some cases the effects found have been due to non-specific toxicity of the ODN and not due to sequence-specific effects of the ODNs used.

4.1.1 Effect of AS1 and AS2 on Cellular Growth of A431 Cells.

A431 cells were treated for 4 hours with the relevant ODN lipid complex at 37°C in the presence of serum-free media followed by a further 20 hours incubation in fresh serum-free medium prior to analysis by trypan blue exclusion or crystal violet assay as described in section 2.8.

Figure 4.1: Effect of AS1 21mer concentration on A431 cell growth over 24 hours.
AS1 ODN was complexed with lipofectin™ (LP) and added to A431 cells in the presence of serum-free media in either 24 well plates or 96 well plates over 24 hours at 37°C and assayed for cell growth as described in section 2.8.1.2 by crystal violet assay and confirmed by trypan blue exclusion (2.8.1.1). All treatments were in the presence of 7.5µM lipofectin™ with the cell number being expressed as percentage control of untreated cells. Data represents the mean n=4 mean ±SD. * represents a significant difference (p<0.05) from the control.

The above results show that increasing doses of AS1 gives a decrease in cell number compared to that of the untreated control cells. Cells treated with lipofectin™ alone did not show any decrease in cell number indicating that the effect shown with AS1 is not simply due to toxicity of the lipid. This is clarified by the results shown in section 3.5 indicating that lipid at the 7.5µM is not significantly toxic to A431 cells when treated over a period of 24 hours. The results show that at 500nM, AS1 causes a 69% (±4%) decrease in control cell number. However, when the dose is increased to 1µM the decrease in cell number is comparable to that found at 500nM. This may be due to the saturation of the lipid: ODN complex at high concentration of ODN thus increasing ODN concentration does not allow more ODN to enter the cell. Saturation of uptake mechanisms has been shown with ODN alone; where the mechanism of uptake is thought to be by a receptor-mediated endocytosis (Loke *et al.*, 1989) thus it is plausible that a

similar problem is probable with lipid-enhanced delivery. As stated in chapter 3 the lipid: ODN ratio is an important factor in ODN uptake, and should be optimised thoroughly. Thus it may be that at higher concentrations of lipid the effect shown with higher concentrations of ODN would be greater. However due to toxicity limitations the lipid concentration cannot be increased any further without non-specific cytotoxic consequences.

From the DNA-chip technology profile it was shown that the most favourable binding for AS1 was at 21 bases and thus this was chosen as the initial length to study. However, to ascertain whether this was an accurate estimation of sequence length and thus to prove the usefulness of DNA chip technology for ODN design a number of other lengths of AS1 were synthesised as described in section 2.2.1 and 3.1 and tested for efficacy on A431 cell growth.

Figure 4.2: Effect of AS1 17mer concentration on A431 cell growth over 24 hours.
AS1 ODN was complexed with lipofectin^{TM} (LP) and added to A431 cells in the presence of serum-free media in either 24 well plates or 96 well plates over 24 hours at 37°C and assayed for cell growth as described in section 2.8.1.2 by crystal violet assay and confirmed by trypan blue exclusion (2.8.1.1). All treatments were in the presence of 7.5μM lipofectin^{TM} with the cell number being expressed as percentage control of untreated cells. Data represents the mean n=4 mean ±SD. * represents a significant difference (p<0.05) from the control.

Figure 4.3: Effect of AS1 14mer concentration on A431 cell growth over 24 hours.
AS1 ODN was complexed with lipofectin™ (LP) and added to A431 cells in the presence of serum-free media in either 24 well plates or 96 well plates over 24 hours at 37°C and assayed for cell growth as described in section 2.8.1.2 by crystal violet assay and confirmed by trypan blue exclusion (2.8.1.1). All treatments were in the presence of 7.5µM lipofectin™ with the cell number being expressed as percentage control of untreated cells. Data represents the mean n=3 mean ±SD.
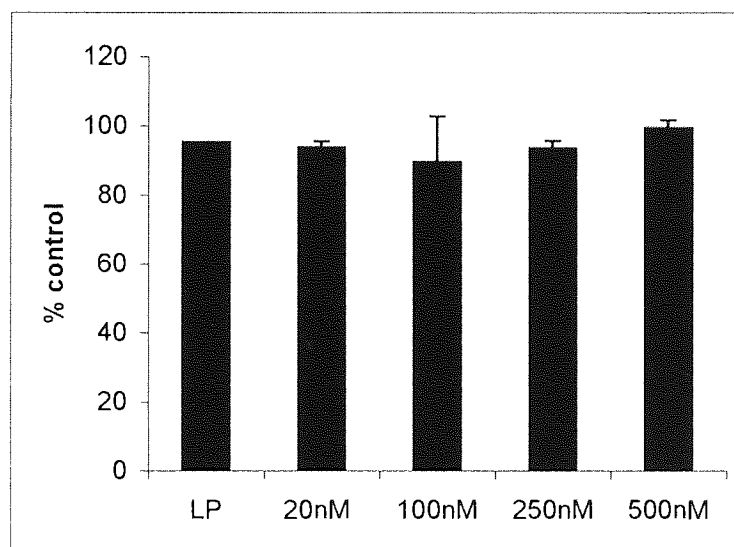
Figure 4.4: Effect of AS1 10mer concentration on A431 cell growth over 24 hours.
AS1 ODN was complexed with lipofectin™ (LP) and added to A431 cells in the presence of serum-free media in either 24 well plates or 96 well plates over 24 hours at 37°C and assayed for cell growth as described in section 2.8.1.2 by crystal violet assay and confirmed by trypan blue exclusion (2.8.1.1). All treatments were in the presence of 7.5µM lipofectinTM with the cell number being expressed as percentage control of untreated cells. Data represents the mean n=3 mean ±SD.

AS1 17mer length had no effect on cell growth at low concentrations (<100nM) and only

32% (±0.3%) reduction compared to control at 500nM. This is less than half the effect

shown at the same concentration for the 21mer AS1. Although a significant reduction (p<0.05) by AS1 was observed on the 17mer length compared to that of 21mer the effect was not so pronounced. However, the 14mer, 10mer and 7mer lengths AS1 has no significant effect (p>0.05) at any dose of ODN tested.
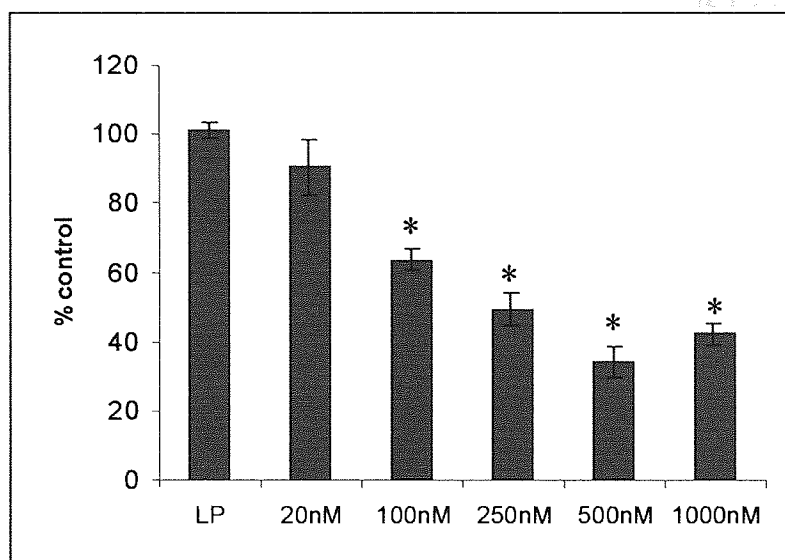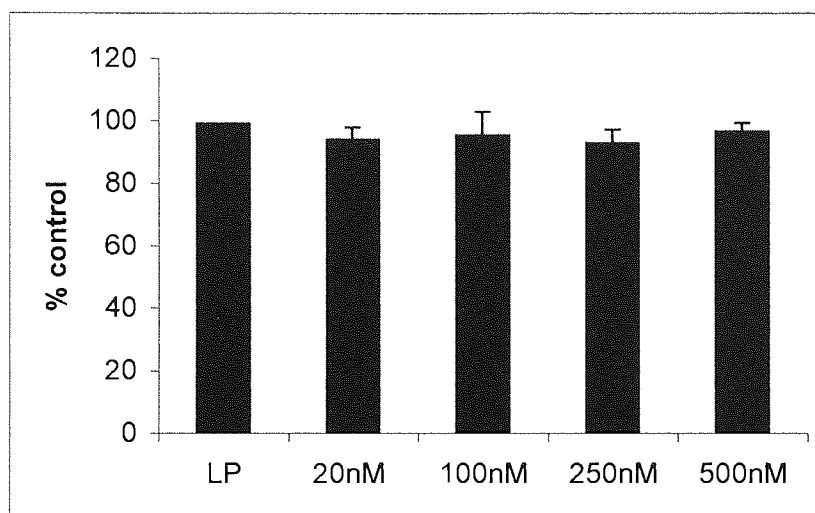

Figure 4.5: Effect of AS1 7mer concentration on A431 cell growth over 24 hours.
AS1 ODN was complexed with lipofectin^{TM} (LP) and added to A431 cells in the presence of serum-free media in either 24 well plates or 96 well plates over 24 hours at 37°C and assayed for cell growth as described in section 2.8.1.2 by crystal violet assay and confirmed by trypan blue exclusion (2.8.1.1). All treatments were in the presence of 7.5μM lipofectin^{TM} with the cell number being expressed as percentage control of untreated cells. Data represents the mean n=3 mean ±SD.

From these studies, it can be shown that as the length decreases the effect of phosphorothioate AS1 on cell growth decreases rapidly with no effect at any dose found using a length of less than 17 oligonucleotides up to 500nM. Although this result is as to be expected it requires some explanation as to why at shorter lengths the effect of AS1 on cell growth is completely lost. As previously stated the delivery of ODN with cationic lipid needs to be optimised for each chemistry, sequence and length of ODN. Thus, although the lipid concentration used here is sufficient for delivery of the 21mer AS1 sequence it may be insufficient for the shorter lengths. However this alone does not explain the dramatic loss of effect at short lengths as the chemistry and sequences of the

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ODNs are equal or very similar. The most plausible explanation is that the length of ODN is important for the mechanism of action of the ODN. As described in chapter one (section 1.1.1.2) there are many methods by which ODNs can inhibit the cell growth, with the activation of RNase H being one such method. Studies have shown that RNase H activation requires both the correct chemistry and a minimum number of bases for activation. It has been shown *in vitro* that 7 bases is the minimum number required for initiation of RNase H to cleave DNA: RNA hybrids in humans and thus degrade RNA and prevent protein production (Wagner *et al.*, 1996). However it appears that variation is evident on the number of bases required dependant on species and on cell type (Sohail *et al.*, 2001). Further to this, work carried out in our laboratory (Hughes, 2001) has shown that RNase H can cleave AS1 at lengths up to 14 oligonucleotides *in vitro* but lengths below this *e.g.* 10mer and 7mer AS1 are incapable of activating RNase H. This would subsequently mean that if the mechanism of action of AS1 were that of RNase H activation, then no cell growth inhibition would occur due to no mRNA degradation occurring. Some mRNA cleavage by RNase H was found with AS1 at 14 bases length with the RNase H assay compared to little effect in cells at this length. This can be accountable by the differences between the two assays in that the RNase H assay is a cell free system assay. Other molecules within the cell such as transcription factors and other proteins may hinder the binding of ODN to mRNA, with smaller ODNs being bound easier by these secondary molecules as they are more accessible, thus preventing their binding to the mRNA and thus activation of RNase H. To confirm the hypothesis that AS1 is acting by an RNase H type mechanism, analysis of mRNA profiles is required both following phosphorothioate AS1 ODN treatment and also treatment with ODNs possessing chemistries that are incapable of activating RNase H. If these ODNs do not show cell growth inhibition then it is postulated that they are not cleaving mRNA due to

their lack of ability to activate RNase H. This issue and that of mRNA profiles are addressed later in this chapter. Furthermore it is conceivable that the shorter length ODNs are not capable of binding to the mRNA as strongly as those of longer sequence. Thus when the ribosome travels along the mRNA sequence translating it to protein, it simply dislodges the small ODN and continues translating the protein, which consequently means cell growth is stimulated by EGFR signalling as normal. This theory concurs with that of steric hindrance mechanisms of action of ODN inhibition of gene expression. This can also be confirmed by the use of mRNA studies as described above.

As the above results show that AS1 21mer (referred to simply as AS1 from here on) gives the most inhibition of cell growth of the lengths studied it was decided that this length should be used for all future work. Although other sequences and chemistries may give different length profiles to those shown here, it is important that all results are comparable and thus the 21mer length was used throughout. As such, similar experiments were carried out using AS2 at 21 bases length. The AS2 sequence was the second of the sequences determined by DNA chip-technology that was predicted to have the potential to inhibit cell growth by binding to the *EGFR* mRNA.
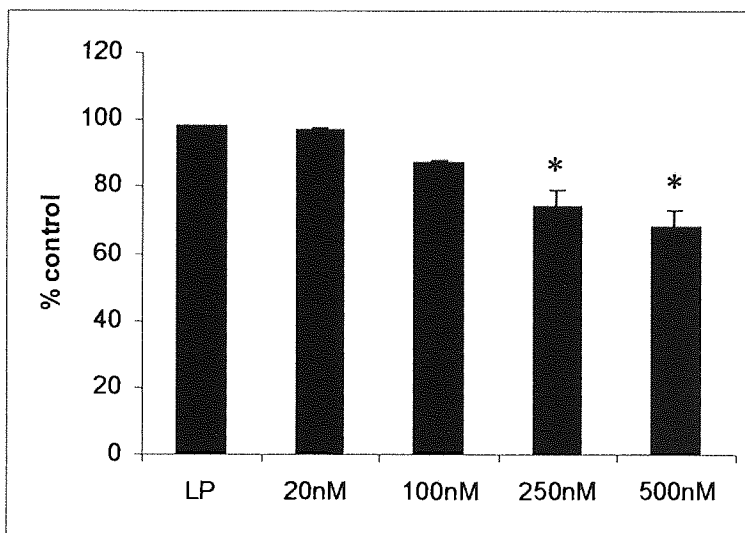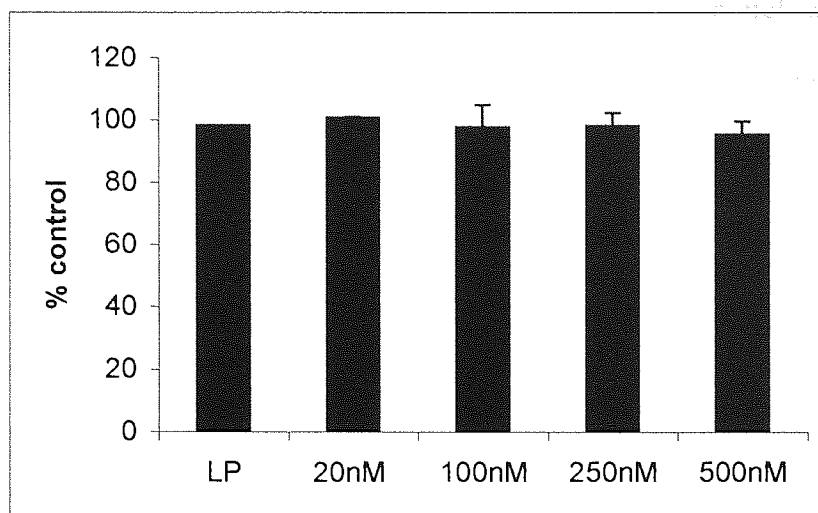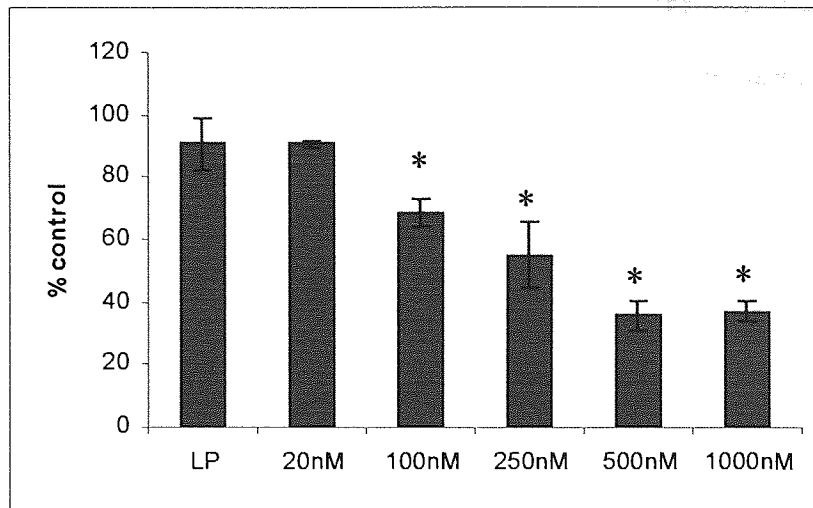
Figure 4.6: Effect of AS2 21mer concentration on A431 cell growth over 24 hours.
AS2 ODN was complexed with lipofectin^{TM} (LP) and added to A431 cells in the presence of serum-free media in either 96 well plates or 24 well plates over 24 hours at 37°C and assayed for cell growth as described in section 2.8.1.2 by crystal violet assay and confirmed by trypan blue exclusion (2.8.1.1). All treatments were in the presence of 7.5µM lipofectin^{TM} with the cell number being expressed as percentage control of untreated cells. Data represents the mean n=4 mean ±SD. * represents significant difference (p<0.05) from the control.

A similar profile to that found with AS1 was observed with a maximum decrease in cell growth of 65% (±4.5%). As with AS1 there was no further increased effect on cell growth at higher concentrations and thus as stated previously this is likely to be due to saturation of uptake mechanism.

As the half-life of the EGFR protein is reported to be between 6-10 hours (King *et al.*, 1980) this would allow 2-4 turnovers in the treatment time. If AS1 were acting at maximal effect then it would be expected that a decrease of 75-100% knockdown of EGFR would be found. The difference found between the theoretical and actual results may be due to many factors. Although the total treatment time was 24 hours it has been shown that uptake of ODN into the cell can plateau after a few hours (Levis *et al.*, 1995; Yakubov *et al.*, 1989) and that even if the ODN is stable for the full treatment time (as shown in chapter 3) the ODN will eventually be transported out of the cell by exocytosis.

Fell, (1999) showed that ribozymes delivered with lipid complex were effluxed from cells shortly after administration with approximately 60% of the ribozyme being effluxed within 4 hours. Thus if it is assumed that a similar profile for ODNs were to be observed then the ODN is not present for the complete 24 hour treatment time to exert its full effect on the cell. Results of delivery studies (Temsamani *et al.*, 1994; Stein *et al.*, 1993) have shown that a burst of ODN is released from the cell within the first hour of treatment probably due to compartmentalisation in early endosome vesicles near to the surface of the cell and that a second phase of efflux is observed over the remaining time due to its deep compartmentalisation in late endosome or lysosome vesicles. In this case the ODN has a reduced time to act at its target and thus will have less effect on the growth rate of the treated cells. Secondly it is plausible that when delivered to a cell population not all cells uptake the ODN (*i.e.* heterogeneous uptake). Untreated cells remaining would likely continue to grow at a normal rate. Finally although the *EGFR* mRNA and thus protein may be inhibited by the effect of ODN treatment it is possible that this alone will not be enough to completely inhibit cell growth. There are many signalling pathways within the cell that allow cell growth and proliferation thus inhibiting just one pathway, that of *EGFR* will not be sufficient to completely inhibit cell growth of a population.

Although the above results seem to indicate that DNA chip-technology is capable of predicting ODN sequences that can have a biological effect, these results alone do not determine whether these effects are sequence specific or simply a non-sequence specific non-antisense effect. Many reports have shown effects induced by ODNs and have stated that they are ascribed to antisense mechanisms (Crooke, 1992; Stein & Cheng, 1993; Wagner, 1994) but only a fraction of these reports have sufficient data to support claims of an antisense specific effect (Monia *et al.*, 1996; Tian *et al.*, 2000; Roh *et al.*, 2000; Wu

et al., 2000). This is considerably more important to establish due to the use of phosphorothioate ODNs as these have been amongst the most popular chemistry shown to exhibit non-specific effects that initially gave false hope for the potential use of antisense therapy in the clinic. In recent years antisense research has begun to become more stringent in the need for adequate controls and the biological effects shown to prove a "true" antisense effect (Crooke, 1996; Agrawal, 1999; Tamm *et al.*, 2001)

4.1.2 Effect of Control PS-ODN Sequences on Cell Growth of A431 Cells.

Various controls have been employed in this study to determine whether the antisense effect found with AS1 and AS2 is sequence-specific. These include scrambled, mismatch sequences and other chemistries. Perhaps one of the most dramatic controls used in this study however is brought about by the use of the design strategy utilised. DNA chip-technology does not only possess the ability to determine and provide sequences that are capable of binding to the target molecule, but also enables us to show regions that are not accessible and thus will not be able to bind antisense molecules. From the chip pattern established at the Department of Biochemistry, Oxford and shown in chapter 3, we were not only able to select sequences AS1 and AS2 but also a sequence found not to be accessible on the *EGFR* mRNA region tested. This sequence was termed AS3 and is perhaps the most novel control to date as it provides proof that the technology has the ability to determine sequences accessible to antisense molecules.

Table 4.1 shows a summary of effects of some of the controls employed at a dose of 500nM only. All controls were delivered in combination with lipofectin^{TM} and as a comparison, results for AS1 alone and in combination with lipid at this dose is also

shown. The use of scrambled sequences is one of the most widely used controls to date and has replaced the traditional sense sequence control. Scrambled sequences help to provide evidence for sequence specificity. The sequence is designed so that the base composition is equal to that of the antisense sequence but the bases are randomly scrambled trying to avoid the types of sequence motifs, such as G quartets, ACTG stretches, AAA and TAA that have been shown to give false antisense effects (Matveeva *et al.*, 2000). Mismatch controls are extremely important in determining specificity of an ODN to its target with the assumption that the greater the number of mismatch bases in the sequence the less chance the ODN will have of binding to its target and exerting its effect. With PS-ODNs much non-specific activity is due to the PS-ODNs having a high affinity for proteins within a cell and thus sequence plays a less significant role in the binding of ODN to the protein (for reviews see Crooke, 1998a; Krieg & Stein, 1995). In this study two mismatch sequences were used for each of AS1 and AS2, containing two and four mismatches in the hope to determine whether the effects observed with AS1 and AS2 antisense sequences are sequence-specific. If PS-ODNs are acting through binding to proteins, inhibiting their effect and thus cell growth, the mismatch sequences would also show an effect particularly at higher base number mismatches.

Treatment used	% Untreated control	SD (±%)
LP only	91	18
AS1 alone (no LP)	98	9
AS1 500nM	31	4
AS1 scrambled	81	0.5
AS1 2 mismatches	39	5
AS1 4 mismatches	78	7
AS2 alone (no LP)	93	5
AS2 500nM	35	5
AS2 scrambled	96	6
AS2 2 mismatches	80	17
AS2 4 mismatches	110	12
AS3 500nM	93	0.8
AS3 5µM	89	7

Table 4.1: Summary of effects of various treatments of AS1 and AS2 on A431 cell growth.
AS1 and AS2 ODNs at 500nM were complexed with lipofectin™ (LP) and added to A431 cells in the presence of serum-free media in either 24 well plates or 96 well plates over 24 hours at 37°C. Assays for cell growth, as described in section 2.8.1.2 by crystal violet assay and confirmed by trypan blue exclusion (2.8.1.1) was performed. All treatments were in the presence of 7.5µM lipofectin™ unless stated, with the cell number being expressed as percentage control of untreated cells. Data represents the mean n=4±SD.

From the results shown in table 4.1 it can be seen that the treatment of A431 cells with scrambled sequences AS1 and AS2 does not have a significant effect on cell growth. Little or no growth inhibition was found compared to the effects found with antisense AS1 and AS2 sequences and the results were similar to those shown with lipofectin™ or ODN alone (no delivery agent). The use of the mismatch sequences shows that the greater the mismatches the less effect on cell growth. An AS1 ODN with two mismatches was able to exert an inhibition of cell growth of 61% (±5%) compared to 69% (±4%) found with antisense AS1 indicating that two mismatches is not sufficient to completely prevent binding of AS1 and its subsequent effects on cell growth in A431 cells. However, four mismatch bases in the AS1 sequence shows effectively no greater effect on cell

growth than control treated cells thus demonstrating that sequence specificity is crucial for AS1 effect. This finding is more pronounced when using AS2, as two mismatches, as well as four, have no significant effect on cell growth indicating that sequence specificity with AS2 is crucial. The sequence specificities found for AS1 and AS2 further demonstrate the specific Watson-Crick base pairing used for antisense binding to its mRNA target and promotes the unique quality of the results obtained using DNA chip-technology as a potential design tool.

Although the above controls are now accepted as standard for antisense experiments we have the unique opportunity to further clarify sequence specificity but also the clarity of DNA chip technology to deduce possible antisense sequences by the use of our third control ODN, AS3. As previously mentioned this ODN was designed not to be able to interact with the mRNA target and thus should show no effect on A431 cell growth mediated via the EGFR signalling pathway. AS3 was added to A431 cells at 500nM similar in dose to those previously used for AS1 and AS2 and the other controls in order to show its effect on cell growth in the presence of lipofectin^{TM}. From the results in table 4.1 it can be shown that 500nM AS3 has no significant effect (p>0.05) on cell growth compared to that seen with AS1 and AS2 at similar concentrations. Furthermore, AS3 when administered at 10 times the dose required to give an effect with AS1 and AS2 does not give any significant effect on cell growth with 89% (±7%) of control cells still remaining after the initial 24 hour treatment in the presence of lipofectin^{TM}. This may however be due to the lack of uptake at higher concentration due to saturation of the lipofectin concentration.

4.1.3 Effect of Different Chemistry AS1 on A431 Cell Growth.

So far cell growth assays performed have all used the phosphorothioate chemistry of ODNs and have shown promising results that indicate a sequence specific effect. However, the method by which cell growth is inhibited by AS1 and AS2 has not been addressed and thus the mechanism of action, although sequence-specific, is still unclear. In order to address this problem a second batch of AS1 and AS2 was synthesised using the 2' O-methyl chemistry. The main differences between these chemistries are discussed in section 1.2.2 but essentially the mechanism of action differs in that PS-ODNs are capable of activating RNase H whereas 2' O-methyl derivatives primarily are thought to act by steric hindrance (Zon, 1988; Inoue et al., 1987). Although these ODNs have shown greater stability in the presence of serum compared to their PS counterparts they have a distinct disadvantage in that they do not allow degradation of the mRNA target (Zon, 1988; Inoue et al., 1987). Although they can stop translation of the mRNA to protein by simply blocking the ribosome from traversing the mRNA sequence, once removed the mRNA is still free to be transcribed. Thus one ODN molecule is only capable of preventing translation of a single mRNA molecule. However, for the purpose described here they are important in helping to determine the mechanism of action by which AS1 and AS2 are acting.

A dose profile similar to that investigated for PS-AS1 and AS2 was investigated for the 2' O-methyl chemistries and as such these ODNs were also delivered with 7.5µM lipofectin^{TM} as used for their PS counterparts.
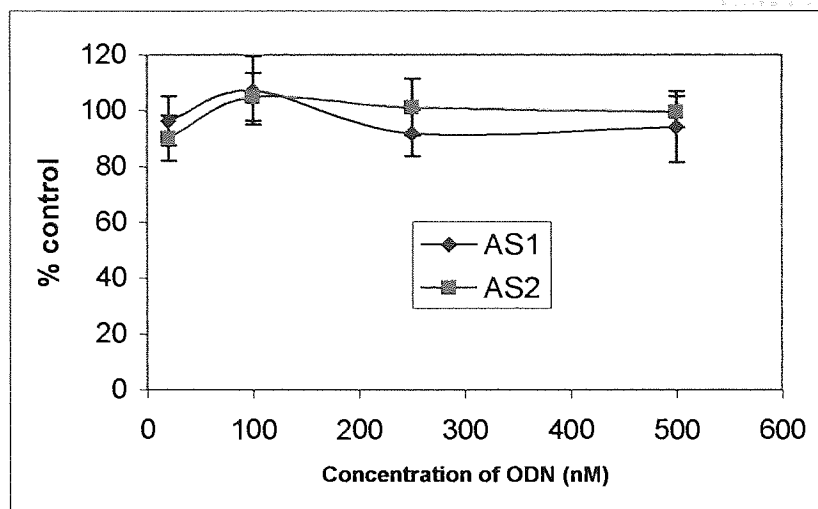
Figure 4.7: Effect of AS1 and AS2 2' O-methyl concentration on A431 cell growth over 24 hours.
AS1 and AS2 ODNs were complexed with lipofectin™ (LP) and added to A431 cells in the presence of serum-free media in either 24 well plates or 96 well plates over 24 hours at 37°C and assayed for cell growth as described in 2.8.1.2 by crystal violet assay and confirmed by trypan blue exclusion (2.8.1.1). All treatments were in the presence of 7.5µM lipofectin™ with the cell number being expressed as percentage control of untreated cells. Data represents the mean n=3±SD.

Figure 4.7 shows that no significant effect (p>0.05) on cell growth was observed when treated with either AS1 or AS2 2' O-methyl ODN over 24 hours at various doses. At the optimal inhibition dose of 500nM it was found that 2' AS1 and 2' AS2 allowed 94% (±13%) and 100% (±5%) respectively of the control cells to remain viable. The lack of inhibition shown using 2' O-methyl derivative indicates that the mechanism of action by which AS1 and AS2 work in the PS form is likely to be via an RNase H type mechanism. If the mechanism of inhibition were simply by steric hindrance then the inhibitory effect of AS1 and AS2 would also be found with the 2' O-methyl chemistry. Although this study provides initial evidence for the mechanism of action of PS- AS1 and AS2, it is not conclusive and further studies to look at the mechanism of action need to be performed. One such way to clarify that RNase H is a contributing factor in this case is to observe any reduction in the total *EGFR* mRNA levels bought about the degradation of mRNA by

the RNase H. This issue is addressed later in this chapter by means of two methods, RT-PCR and RPA analysis.

In summary the data shows that although 2' *O*-methyl derivative ODNs offer the distinct advantage that they are more stable in serum conditions (see figures 3.3 and 3.4) and are deemed less toxic due to their reduced non-specific binding, they have the distinct disadvantage in this case over PS-ODNs in that they cannot activate RNase H. Thus 2' O-methyl ODNs are a saturable method of inhibiting *EGFR* mRNA translation and subsequent cell growth. In A431 cells they are not capable of inhibiting *EGFR* translation and thus are of no use as a potential antisense therapy in these cells.

Effort has now been devoted to combining the two distinct advantages of the 2' *O*-methyl and PS modifications in order to establish a chemistry that can utilise the best of both of these chemistries without the problems / disadvantages associated with them. These ODNs are now termed chimeric ODNs (or mixed backbone ODNs) and consist of both chemistries in one ODN molecule. This has been achieved with a variety of chemistries, however in this case concentration has been aimed at utilising the previously studied PS and 2' *O*-methyl chemistries. It was attempted to make an ODN that would combine both the stability of the 2' *O*-methyl chemistry but allow the RNase H activation ability of the PS ODN. In terms of stability most ODN degradation occurs due to exonucleases attacking the ends of the molecule, thus the 3' and 5' ends of the ODN were constructed of 2' *O*-methyl residues. RNase H activation simply requires a stretch of bases that are PS chemistry and thus a central area of the molecule was constructed of PS residues.

When considering the precise sequence of the ODN it is necessary to ensure that the sequence of PS residues is of sufficient length to activate the RNase H. As previously

mentioned work in the field of RNase H activation has shown many variations in the minimum number of bases required to perform this. As the binding of PS residues to proteins is that which causes non-specificity in ODN treatments it was decided to keep to the minimum number possible to ensure RNase H activation but to minimise non-specific binding. The previous length study showed that a minimum of a 17mer AS1 length was needed to obtain an effect on cellular proliferation. Various lengths of PS bases within the chimeric sequence were tested in the laboratory for activation of RNase H. It was found with the 2' O-methyl flanks only seven PS bases were required to give RNase H activation (Hughes, 2001). The assay showed that the chimeric AS1 (sequence shown in table 3.1) was capable of cleaving RNase H and thus would be suitable to test for its effect of cell growth.
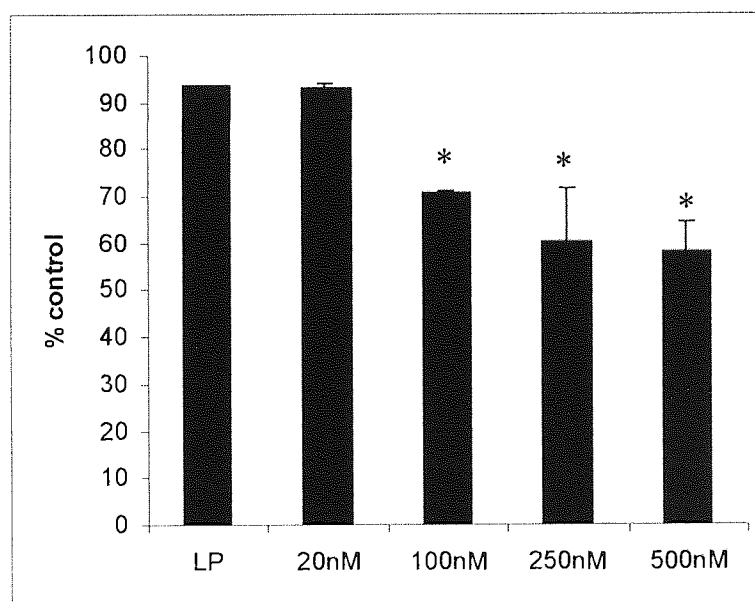
Figure 4.8: Effect of chimeric AS1 on A431 cell growth over 24 hours when administered in the presence of lipid.
Chimeric AS1 ODN was complexed with lipofectin™ (LP) and added to A431 cells in the presence of serum-free media in either 24 well plates or 96 well plates over 24 hours at 37°C and assayed for cell growth as described in 2.8.1.2 by crystal violet assay and confirmed by trypan blue exclusion (2.8.1.1). All treatments were in the presence of 7.5μM lipofectin™ with the cell number being expressed as percentage control of untreated cells. Data represents the mean n=3±SD. * represent a significant effect (p<0.05) compared to that of the control.

From the results obtained it appears that the chimeric ODN designed has some effect on cell growth in a dose profile similar to that of PS-AS1. At 500nM the chimeric ODN

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gives an inhibition of cell growth of 42% (±14%) and although this is not as great an effect as that of PS-AS1 it does suggest that the chimeric profile is a suitable way of utilising the increased stability of 2' O-methyl residues without the loss of the RNase H activation accustomed to that of the PS chemistry. Loss of antisense effect found with the chimeric ODNs compared to PS-AS1 may be accounted for by the lack of optimisation for this specific ODN. The optimisation of delivery and chemistry is beyond the scope of this work as the concentration is not on these issues but more on design and evaluation. However it is possible with further evaluation on precise number of PS residues as well as increased optimisation on delivery strategies that this type of ODN could be much more widely used in the future of antisense technology. In this case, the A431 cells are capable of surviving in a serum-free environment thus the use of 2' O-methyl chemistry is not vital to give stability advantages, however in a clinical situation this therapy would be much more useful where stability would be an issue.

With thought to the use of these ODNs in future therapy, it is important to establish the number of treatments that may be required for the complete eradication of the tumour cells in any given tumour. So far all of the treatments used have been with one dose in 24 hours and have given the desired effect within that period. However complete knockdown is not achieved by this dose and thus the remaining cells could continue to grow unless the effect seen by the ODN is preventing the cells from growing e.g. cell cycle arrest but not actually killing the cells. In order to determine the effect of the ODN over time it is important to test whether the effect of the ODN is irreversible and thus the cells will resume their normal growth pattern over time. In a review by Stein, (1999) it is stated that the reversibility of an antisense effect can be seen as another control in the proof of an antisense mechanism. If a gene is knocked out in a cell line over-expressing that gene (as

is the case with A431 cells) and the cells are rescued from the effect it strengthens the case for an antisense effect, although does not prove it.

A431 cells were treated as before with 500nM AS1/ AS2 complexed with lipid for 4 hours and then fresh media added. Cells were then counted over 120 hours to determine whether the cells would resume growth or continue to die.

A)
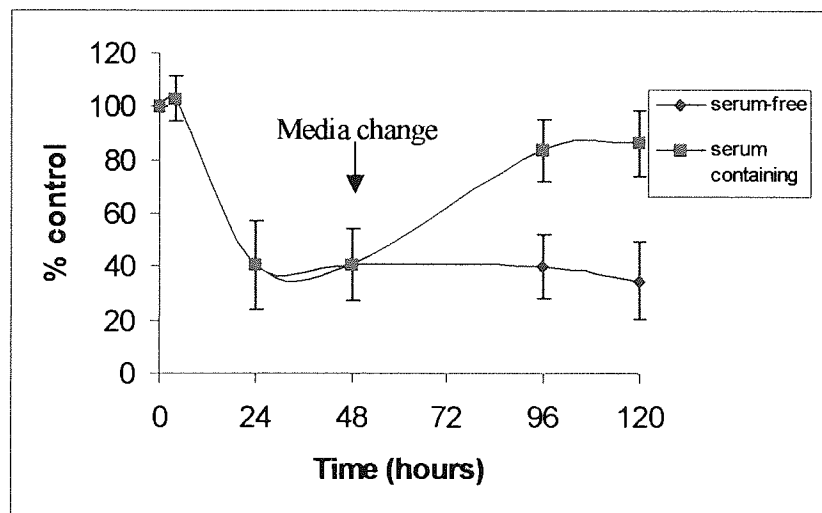
B)
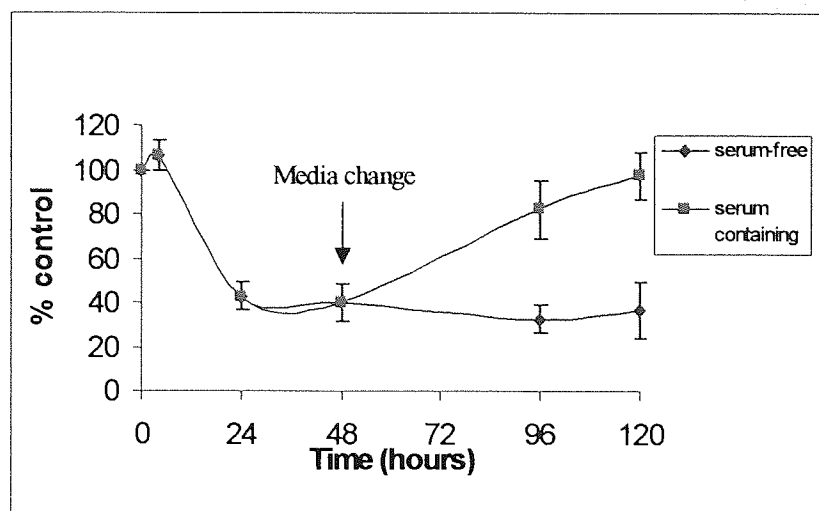
Figure 4.9: Effect of AS1 and AS2 on A431 cell growth over 120 hours when administered in the presence of lipid.
A) AS1 and B) AS2 ODN were complexed with lipofectin™ (LP) and added to A431 cells in the presence of serum-free media in either 24 well plates or 96 well plates at 37°C and assayed for cell growth as described in section 2.8.1.2 by crystal violet assay and confirmed by trypan blue exclusion (2.8.1.1). After 48 hours the media was changed to either fresh serum-free or complete media containing 10% FBS. All treatments were in the presence of 7.5µM lipofectin™ with the cell number being expressed as percentage control of untreated cells. Data represents the mean n=3±SD.

As shown in figures 4.9A and 4.9B, the cells do not resume their normal growth rate for the following 120 hours after treatment in the presence of serum-free media. However, this can be attributed not to the irreversible toxic effects of the ODN to all of the cells but more so to the lack of nutrients / serum within the media after this period. It can be clearly seen in both cases that when serum-containing media is added after 48 hours, the cells begin to resume their normal growth characteristics and within a further 72 hours are near to their original cell number. It is postulated therefore that although A431 cells are capable of growing at a normal rate in serum free media for 24 hours as shown in figure 3.14 it is clear that after this time and in conditions of stress such as that following ODN treatment, the cells require an influx of nutrients to resume their normal cellular growth cycle. Studies such as this also provide information regarding possible treatment regimes for complete regression of a tumour cell population. It is clear from these results

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that although one ODN dose is sufficient to decrease cell growth it is not sufficient to eradicate the cells completely thus further treatments would be required for complete reduction in cells. It is clear that the cells are only capable of sustaining their own growth in serum-free culture for approximately 48 hours and thus the need for re-addition of serum media following this time to allow culture re-growth.

Perhaps the most important aspect of reversibility of ODN effect comes when considering future use in the clinic. It is inevitable that when treating a tumour some of the surrounding normal tissue is going to be affected by the treatment although delivery strategies would hope to minimise this risk. Once tumour regression / removal has been established it is important to consider the effect of any residual ODN on normal cells that still remain in the target area. It is important, especially in the brain where new cell growth is rarely found, that these cells can recover their cell growth characteristics and continue to function in their normal role. Thus by ensuring reversibility of any effects on the cell once the ODN is removed is necessary for this to occur. It should be considered however that this reversibility also brings about implications for the design of individual tumour treatments to ensure that the entire tumour is killed before cessation of treatment. If there are some tumour cells remaining it is plausible that these can reverse the effects of the ODN and continue to divide enabling reoccurrence of the tumour.

In summary we have shown that DNA chip designed ODNs are capable of inhibiting cell growth in a sequence-specific manner with control sequences showing little or no effect in comparison to AS1 and AS2. In conjunction with this the chemistry of the ODN is important in determining whether an effect is seen indicating that an RNase H type mechanism is involved and required for cell growth inhibition to occur. Finally, it has been shown that although a significant effect can be found with AS1 and AS2 it is

possible to reverse this effect provided that the correct nutrients and growth factors are available for the cells to divide and grow.

4.2 Effect of Antisense and Control ODNs on Protein Expression Levels in A431 Cells.

4.2.1 Optimisation of Western Blotting

In order to determine any effect of ODN on protein expression levels it is necessary to optimise the western blotting procedure. Thus prior to commencing experiments to determine whether the DNA chip designed ODNs have any effect on EGFR protein expression levels, the protein levels of whole cell lysates required to obtain a band representing EGFR protein of detectable intensity, were performed. Lysates were prepared of untreated A431 cells and subsequently loaded onto SDS-PAGE gels in increasing amounts of total protein. Gels were then subjected to immunoblotting for EGFR and actin protein levels prior to analysis of band intensity by densitometry (section 2.9).

a)

Total Protein (µgs)⇒	2	4	6	8	10	15	20	30

EGFR (170kDa)⇒

Actin (27kDa) ⇒

b)
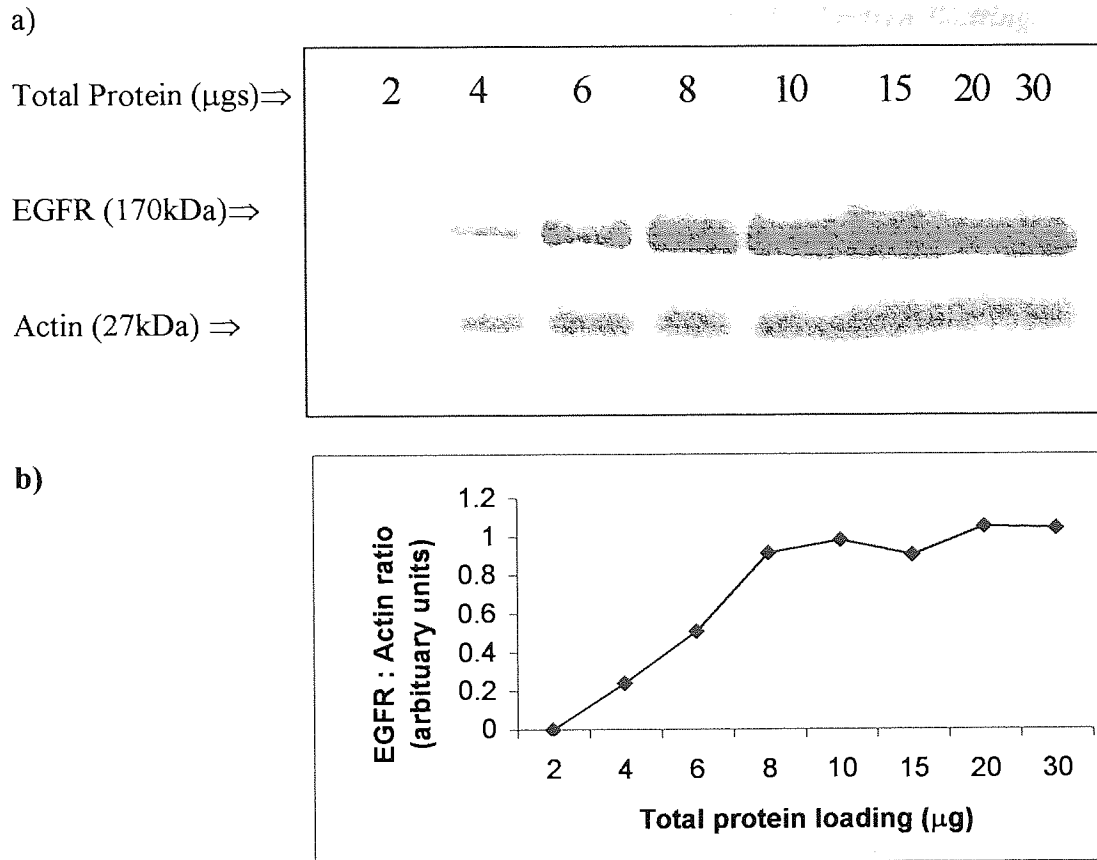
Figure 4.10: Optimisation of total protein loading for western blotting of EGFR protein.
Cells were harvested and lysed as in section 2.9.1 from 24 well plates and analysed for both cell number and protein amounts. Samples were loaded on SDS-PAGE gels at increasing concentrations and subjected to separation before transfer to nitrocellulose membrane and immunoblotted for EGFR and actin protein levels. a) Western blot image; b) graphical representation of relationship between actin, EGF and band intensity.

It can be observed from these results that at high levels of total protein, the limit of detection is reached with the trend becoming a plateau. Conversely at low levels the EGFR cannot be detected as clearly as that of actin. It is important that a linear relationship is established between protein amount and band intensity so that a comparison of effect between bands can be made. For this reason it was decided to use a maximum loading of 8µg of total protein thus allowing any reduction in protein levels to be visible as bands but for high levels, such as those in the control, to be in the linear range.

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4.2.2 Inhibition of EGFR Protein Levels Over 24 Hours by Western Blotting.

Previous results showed a significant reduction in cell number of A431 cells in the presence of antisense ODNs AS1 and AS2 when delivered with optimised lipid complex. As these findings were shown only to occur with the antisense ODNs and not the control ODNs it is postulated that the inhibition in cell number is a result of *EGFR* inhibition thus leading to a knockout of the EGFR signalling cascade that would normally stimulate cell growth. To determine whether this is true, it is necessary to establish a decrease in EGFR protein expression, which would normally occur as a result of *EGFR* mRNA expression inhibition by molecules such as antisense ODNs. It has been postulated that along with the proper control ODN sequences, a change in protein expression levels, specific to the protein of interest is an important factor in determining antisense sequence specificity (Stein, 1999).

As cell growth experiments showed that one dose of ODN in 24 hours was sufficient to inhibit approximately 60% of cell growth it was postulated that this would also be sufficient to show a decrease in protein expression levels. EGFR protein half-life in fibroblasts is approximately 6-10 hours (King *et al.*, 1980) and thus within 24 hours it is expected that the protein level could be reduced by approximately 75-100%.

Cells were incubated with lipid complexed AS1 and control ODNs for 4 hours followed by fresh serum-free media for a further 20 hours. Lysates were then prepared and stored at −20°C prior to use. Cell number was also counted as well as total protein amounts measured to analyse the concentration of protein present in each lysate. This enabled approximately equal amounts of protein to be loaded for each sample thus allowing a

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direct comparison of protein levels and the effect on these by ODN treatment. Western

blot analysis was performed to enable EGFR protein expression level to be observed as

well as those levels of actin protein to ensure equal loading and specificity of effect to

EGFR.
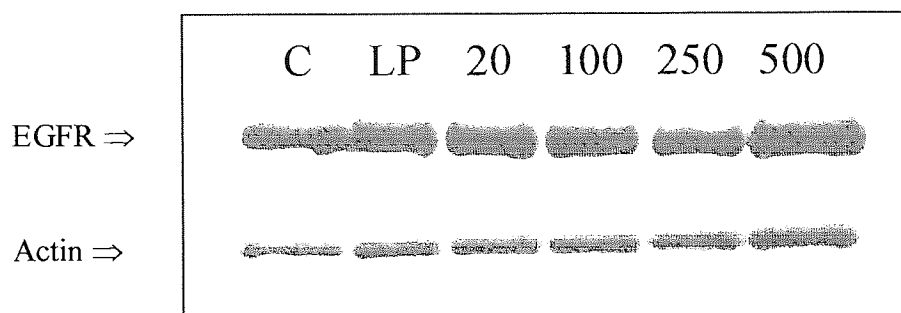
Figure 4.11: Effect of AS1 on EGFR protein expression level in A431 cells over 24 hours.
A431 cells were plated in 24 well plates and cultured for 24 hours in the presence of AS1
complexed with lipid after which period protein expression levels were determined by western
blot analysis. After 24 hours cells were lysed and total cell lysates (8μg per well) analysed by
7.5% SDS-PAGE and immunoblotting using either EGFR or actin antibodies (section 2.9.5).
Image depicts representative blot of triplicate experiments. C represents control untreated lysates
and LP represents lipofectin™ only treated cells.

The results clearly show that there is no striking difference between protein expression

levels with different doses compared to those found in control samples. Although the

loading is not exactly equal throughout the samples (as seen by variations in actin

expression levels), it is clear that these variations are not large enough to indicate any

overall change in EGFR expression levels due to treatment with AS1 ODN.

Upon considering these results it was decided that although in principle one dose within

24 hours should be sufficient to establish an effect in protein expression levels, it could

be that subsequent doses are required and / or longer incubation periods. As it takes the

ODN approximately four hours to enter the cells and reach its target there are only 20

hours remaining for the ODN to inhibit protein expression levels. Within this time it is

possible that some protein is still being expressed and that the EGFR antibody will still detect this pool of EGFR within the cell. Thus, although the ODN will start to inhibit any further *EGFR* expression it will not degrade any protein already formed which may mask the effect being shown by the ODN on protein levels. By increasing the duration of the experiment it is hoped that the EGFR already present will have been degraded via its normal pathway and that the effect of the ODN will be evident. However, by simply increasing the duration and not the number of doses of the ODN is not sufficient as the ODN firstly will start to be degraded within the first 24 hours and thus allow the cell to start to recover for the following 24 hours and secondly the ODN is transported out of the cell within this time thus is no longer able to elicit a response. In order to show the latter, efflux studies would need to be performed but due to time constraints and previous studies (Fell, 1999) these were not investigated in this instance.

4.2.3 Inhibition of EGFR Protein Levels by ODNs Over 48 Hours.

a) Effect of Multiple Doses of AS1 on Cell Growth Over 48 Hours.

With the above information in mind, the experiment was repeated but over 48 hours with a repeated administration of ODN to the cells at 24 hourly intervals, giving two doses over the 48 hours. As so far all cell growth experiments have been performed using one dose over 24 hours, a cell growth assay was performed to see firstly the effect of the extra ODN on cell number over the time and secondly to ensure that the second dose of lipofectin^{TM} would not be toxic to the cells.
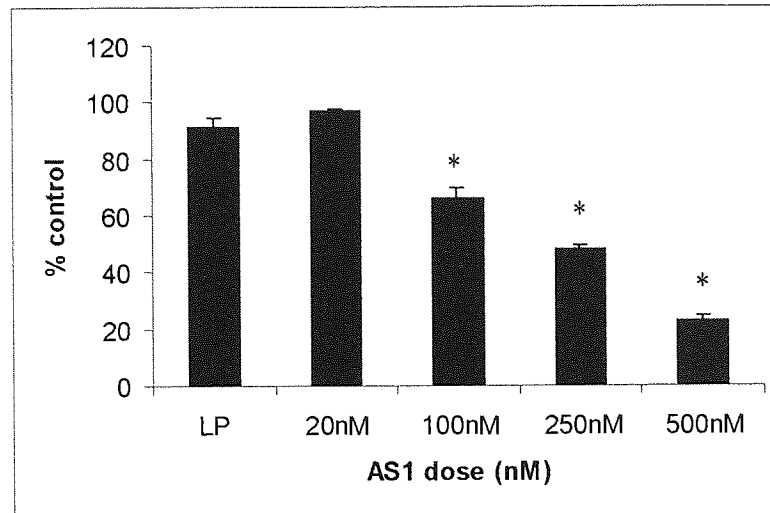
Figure 4.12: Effect of multiple doses of AS1 on A431 cell growth over 48 hours.
AS1 was complexed with lipofectin^{TM} (LP) and added to A431 cells in the presence of serum-free media in either 24 well plates or 96 well plates over 48 hours at 37°C and assayed for cell growth as described in section 2.8.1.2 by crystal violet assay and confirmed by trypan blue exclusion (2.8.1.1). All treatments were in the presence of 7.5μM lipofectin^{TM} with the cell number being expressed as percentage control of untreated cells. Data represents the mean n=3 mean ±SD. * represent a significant difference (p<0.05) to that of the controls.

The results show that the effect of AS1 over 48 hours with repeated doses is slightly more effective than a single dose AS1 over 24 hours but not as much as expected. At 500nM two doses of AS1 produced an inhibition in cell growth of 78% compared to that of 69% found at 24 hours. However, the important result to take from this experiment is that LP alone does not confer any significant toxicity on the cells over the period of the experiment when given in two 7.5μM doses compared to that found with one dose over 24 hours. This indicates that is plausible to treat the cells with two doses of ODN and lipid complex over 48 hours to determine whether an effect on EGFR protein expression levels can be observed.

b) Effect of AS1 on EGFR Protein Levels Over 48 Hours with Multiple Doses.

a)

EGFR (170kDa)⇒

Actin (27kDa)⇒
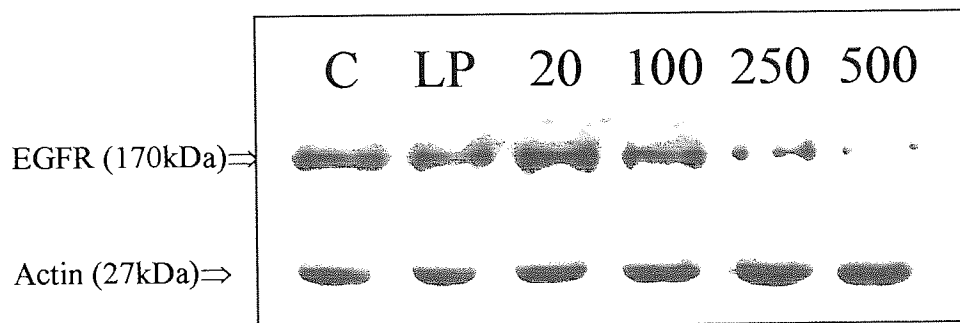
b)
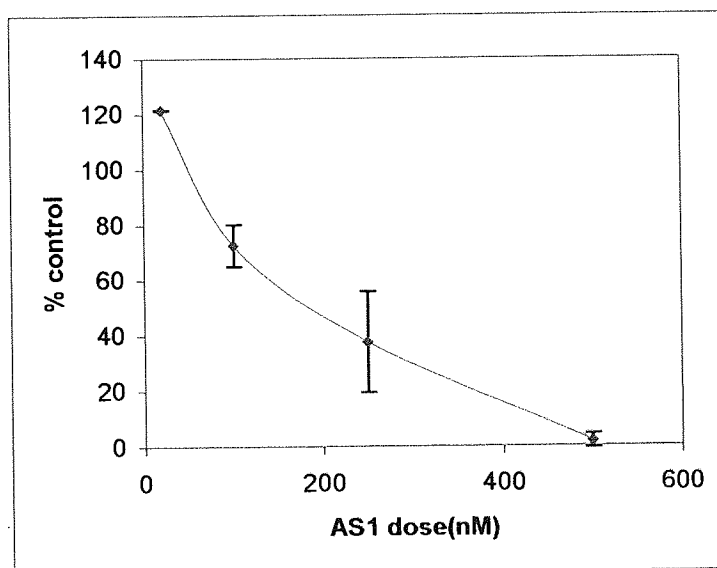
Figure 4.13: Effect of multiple AS1 doses on A431 cell EGFR protein expression levels over 48 hours.
A431 cells were plated in 24 well plates and cultured for 48 hours in the presence of AS1 complexed with lipid after which period protein expression levels were determined by western blot analysis. After 48 hours cells were lysed and total cell lysates (8μg per well) analysed by 7.5% SDS-PAGE and immunoblotting using either EGFR or actin antibodies (section 2.9.5). a) Image depicts representative blot of triplicate experiments b) represents analysis by densitometry. C represents control untreated lysates and LP represents lipofectin™ only treated cells.

The above immunoblot (figure 4.13a) clearly shows that there is a decrease in protein levels with increased amount of AS1 with the levels of actin remaining fairly constant throughout. To ensure that the difference in levels of protein was truly due to the antisense treatment a ratio of actin to EGFR levels was performed to account for equal

loading of total protein levels. This assumes that the levels of actin protein are unchanged by the AS1 but is a fairly safe assumption as there was found to be no occurrence of the AS1 target sequence in actin by a BLAST search. As this is the case it can be assumed that AS1 is having the desired effect on EGFR protein levels of A431 cells. The effect shows a significant decrease ($p<0.05$) in EGFR at both 250nM and 500nM of 62% ($\pm18\%$) and 98% ($\pm3\%$) respectively.

C) Effect of Control ODN Sequences on EGFR Protein Levels over 48 Hours with Multiple Doses.

As with the cell growth assays it is important to establish that control ODN sequences have little or no effect on EGFR protein levels so as to indicate that the effect seen is truly sequence specific. As with AS1, cells were treated with two doses of 500nM ODN over 48 hours and the protein levels established of both EGFR and actin by immunoblotting technique.

a)

| C	LP	Scr	AS3	2'o-me	mis 2	
						EGFR (170kDa)
						Actin (27kDa)
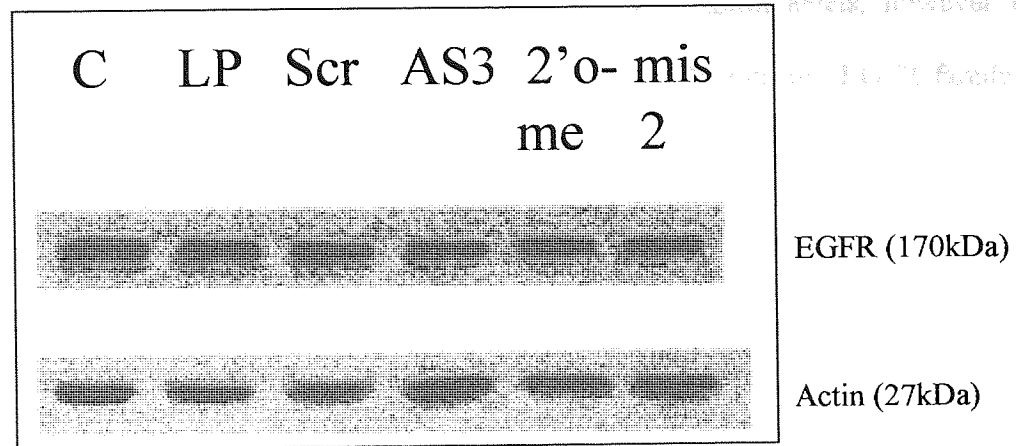
b)
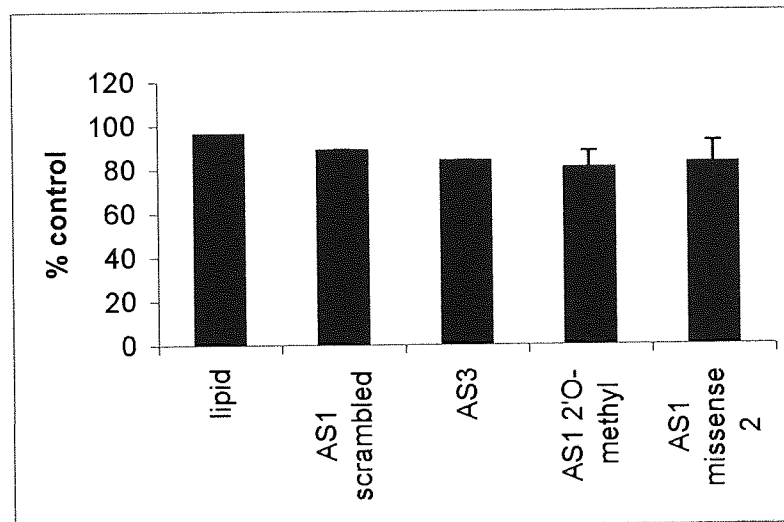
Figure 4.14: Effect of various control ODNs on EGFR protein expression levels over 48 hours.
A431 cells were plated in 24 well plates and cultured for 48 hours in the presence of AS1 complexed with lipid after which period protein expression levels were determined by western blot analysis. After 48 hours cells were lysed and total cell lysates (8μg per well) analysed by 7.5% SDS-PAGE and immunoblotting using either EGFR or actin antibodies (section 2.9.5). a) Image depicts representative blot of triplicate experiments b) represents analysis by densitometry. C represents control untreated lysates and LP represents lipofectin[TM] only treated cells. Scr = scrambled AS1, 2'O-me = 2' O-methyl AS1 and 2 mis = 2 base mismatch AS1.

The results shown in figure 4.14 indicate that none of the controls investigated showed any significant effect (p>0.05) on EGFR protein expression levels over 48 hours. Although these results along with the cell growth results seem conclusive in that the AS1 ODN is clearly having an effect and that this seems to be an antisense effect, it is important to establish whether any other proteins are affected by the ODN. It has already

been shown that AS1 has no effect on actin protein expression levels, however the sequence homology between these two proteins is minimal. Within the EGFR family of proteins there is a significant amount of sequence homology and thus to really test the specificity of AS1 it is necessary to look at these proteins as well. It is possible to look at the homology between family members of proteins using sequence obtained from the ncbi website (http://www.ncbi.nlm.nih.gov/BLAST) and using computer software programs such as Bioedit (http://jwbrown.mbio.ncsv.edu/BioEdit/bioedit.htm) and as such the homology between *EGFR* (*ERBB1*) and *ERBB2*, *ERBB3* and *ERBB4* was determined.

4.2.4 The Sequence Homology of ERBB Family Members and the Inhibition of ERBB2 Protein Levels by AS1.

As members of the *ERBB* family, it is clear that there must be some similarities between the members namely, *ERBB1*, *ERBB2*, *ERBB3* and *ERBB4*. When inhibiting one of the above family members it is important to qualify the effect on other family members especially when dealing with a sequence specific approach. It is evident from sequence data that there will be some homology between the family members in order for them to qualify as a family of proteins and as such it is important to determine whether this homology will have any bearing on the actions of the ODN.

Data shown in figure 4.15 shows the homology between the four *ERBB* family members with gaps indicating homology to *ERBB1* (*EGFR*) sequence. Data given by BioEdit indicates that there is 42% homology between *ERBB1* and *ERBB2* when aligned at the start codon. However, the homology found between AS1 and *ERBB2* is even more

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striking. The homology of AS1 to *ERBB1* is obviously 100% as this is the sequence to which it was designed. However the homology between AS1 and *ERBB2* is shown to be 57%, suggesting there is a possibility that the AS1 sequence could bind to *ERBB2* and thus elicit an antisense effect similar to that shown with *EGFR*.

Due to these findings it was decided to determine the effect of AS1 on *ERBB2* protein levels by western blot analysis. Although it is expected that some effect may be shown it is hopeful that the effect seen will be less dramatic as that seen with *EGFR* due to the reduced sequence homology.

```
                    10         20         30         40         50         60
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
c-erbB1.  atgcgaccct ccgggacggc cggggcagcg ctcctggcgc tgctggctgc gctctgcccg
c-erbB2   ...gag.tgg .g.cctt.tg .c.ctgg.g. .....cctcg cc..cttgc. c.c.g.ag.c
c-erbB3   ...a.gg.ga a..acg.tct gca..tgct. gg.t..cttt .cagcctg.. c.ggg..t.c
c-erbB4   ...aag..gg .gacaggact tt...tctg. g.gagcctt. .cg....g.. .gggac.gtc

                    70         80         90        100        110        120
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
c-erbB1.  gcgagtcggg ctctggagga aaagaaagtt tgccaaggca cgagtaacaa gctcacgcag
c-erbB2   .....caccc aag..tgcac cggc.c..ac atga.gctgc g.ctccctgc cagtc.cg..
c-erbB3   .a.gtgg.ca actctc...c .gt.tgtcc. g.gactctg. atg.cctg.g tg.g..cggc
c-erbB4   ca.ccca.c. a.tctc..tc .gt.tgt.ca g.aacg.ag. at.aactg.g ctctctctct

                   130        140        150        160        170        180
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
c-erbB1.  ttgggcactt ttgaagatca ttttctcagc ctccagagga tgttcaataa ctgtgaggtg
c-erbB2   accca.ctgg acatgctc.g ccac...ta. .agggct.cc a.g.ggtgc. gg.aa.cc..
c-erbB3   gat.ctgaga acc..t.c.. gaca..gta. aag.tctacg a.aggtg.g. ggtg.t.a..
c-erbB4   gacctggaac agc.gt.c.g agcct.gc.. aagt.ctatg aaaa.tg.g. ggt..tca..

                   190        200        210        220        230        240
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
c-erbB1.  gtccttggga atttggaaat tacctatgtg cagaggaatt atgatctttc cttcttaaag
c-erbB2   .aa..cacct .cc..ccc.c c.atgccagc .t.tccttcc tgc.gga.at .caggaggt.
c-erbB3   .ggaaccttg .ga.t.tgc. c..gggacac a.tgccg.cc tctcct.cct gcag.gg.tt
c-erbB4   .g.aacct.g .ga.aacc.g c.ttg.gcac a.cc..g.cc tctcct.cct gcgg.ctgtt

                   250        260        270        280        290        300
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
c-erbB1.  accatccagg aggtggctgg ttatgtcctc attgccctca acacagtgga gcgaattcct
c-erbB2   caggg.t.c. t.c.catc.c .c.caa..aa g.gagg.agg t.c..c..c. .a.gc.g.gg
c-erbB3   cgagaagt.a ca.gcta..t cctc..ggc. ..gaatgaat t.t.tactct a.c.t.g..c
c-erbB4   cgagaagtca ca.gctac.t g.ta..ggct c..aat.agt ttcgttacct ..ctc.ggag

                   310        320        330        340        350        360
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
c-erbB1.  ttggaaaacc tgcagatcat cagaggaaat atgtactacg aaaattccta tgccttagca
c-erbB2   a.t.tgcgag gcacccagc. .ttt.agg.c .ac..tgc.c tggccgtgct a.a.aat.g.
c-erbB3   aacctccg.g ..gt.cgagg g.cccaggtc tacg.tggga .gtt.g..at ctt.g.catg
c-erbB4   aattt.cg.a .tattcgtgg g.c.aa.ct. tatg.gg.tc g.t..g...t g...aa..ttt

                   370        380        390        400        410        420
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
c-erbB1.  gtcttatcta actatgatgc aaataaaacc ggactgaagg agctgcccat gagaaattta
c-erbB2   .a.ccgctg. ...a..acca. ccc.gtc..a ..ggcctccc caggagg.c. .c.gg.gc.g
c-erbB3   t.gaac.a.. ..acca.ct. c.gcc.cg.t ctg.gcc..c tc.gcttg.c tcagctcacc
c-erbB4   t.aaac.ac. gaa.a....g ...ctttgga ctt.aag.ac ttggatta.a ..acttgac.

                   430        440        450        460        470        480
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
c-erbB1.  caggaaatcc tgcatggcgc cgtgcggttc agcaacaacc ctgccctgtg caacgtggag
c-erbB2   ...cttcgaa gc.tcaca.a ga.ctt.aaa g.aggggt.t tgat...a.c. g...cccc..
c-erbB3   g..attc.gt caggg..t.t ttatattgag .ag...g.ta agctttgtca ..tg.acaca
c-erbB4   g.aatcc.aa atgg...a.t .tatgtaga. cag.....at tcctttgt.a tgca.acacc
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```
              490        500        510        520        530        540
         ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
c-erbB1. agcatccagt ggcgggacat agtcagcagt gactttctca gcaacatgtc gatggacttc
c-erbB2. ct.tg.t.cc a.gacacg.. tt.gt.g.ag ...a.ct..c a...g.acaa cca.ctggct
c-erbB3. .ttga.tg.a ..gacat.g. gaggga.c.a ..tgc.gag. tagtgg..aa .gaca.tgg.
c-erbB4. .ttcattg.c aagatattg. tcgg.a.cca tggcc.tc.. a.ttg.ctct tg..tcaaca

              550        560        570        580        590        600
         ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
c-erbB1. cagaaccacc tgggcagctg ccaaaagtgt gatccaagct gtcccaatgg gagctgctgg
c-erbB2. .tc.ca.tga .a.a..c.aa ..gctctc.g .cctgcca.c cctgttc.cc ..tg..taa.
c-erbB3. aga.g.tgt. cccc.t.tca tg.ggtt...c a.ggggc.a. .ctgggg.cc tg.a.cagaa
c-erbB4. a.tggtagtt ca...at.tg. a.gttgcca. a.gt.ct.ta c.gg.cg.t. ct.gg.accc

              610        620        630        640        650        660
         ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
c-erbB1. ggtgcaggag aggagaactg ccagaaactg accaaaatca tctgtgccca gcagtgctcc
c-erbB2. ..ct.cc.ct gctg.ggaga gagttctga. gattgtcag. g.ctga.g.g cactgt..gt
c-erbB3. .actgccaga catt..c.aa gacc.tctgt g.tcctcagt gtaa..gt.. ctgc.ttggg
c-erbB4. aca.a.aatc .ttgcc.gac ttt..c.ag. ..ggtgtgtg cagaacaatg tg.cg..aga

              670        680        690        700        710        720
         ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
c-erbB1. gggcgctgcc gtggcaagtc ccccagtgac tgctgccaca accagtgtgc tgcaggctgc
c-erbB2. .ccg.tg..t ...c.cgc.g .aagg.gcca ctgcc.actg ..tgc..cca ..agcag..t
c-erbB3. cccaa.cc.a accagtgc.g ..atga...g ..tgc.gggg g.tgc.ca.g cc.tcagga.
c-erbB4. t.cta.g.a. c.ta.gtcag tga.t.ctg. catc.ag.at gtgctg.a.g ctgctcag.a

              730        740        750        760        770        780
         ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
c-erbB1. acaggcccc gggagagcga ctgcctggtc tgccgcaaat tccgagacga agccacgtgc
c-erbB2. g.t.c.gg.t .cac.g..cc .aag.actct ga.t..ctgg c.t.cct.c. ctt..acca.
c-erbB3. ....a.tg.t tt.cct..cg gca.t.caat ga.a.tgg.g c.t.t.tacc tcg.tgtcca
c-erbB4. c.taagga.a ca...ct..tt tgc.tgca.g aattt...tg a.a.t.ga.c .tgtgttact

              790        800        810        820        830        840
         ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
c-erbB1. aaggacacct gcccccaact catgctctac aaccccacca cgtaccagat ggatgtgaac
c-erbB2. .gt.g..t.. .tgag.tg.a .tgc.cagc. ctggt....t aca..acaga cacgt.tg.g
c-erbB3. c..cctcttg t.ta.aacaa gc.aact.t. c.g.tggaac .ca.t.ccca caccaa.t.t
c-erbB4. c..tgtc..c aaa...tttg. .tacaatcca .c.a..tttc aactgg..ca ca..t.c..t

              850        860        870        880        890        900
         ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
c-erbB1. cccgagggca aatacagctt tggtgccacc tgcgtgaaga agtgtccccg taattatgtg
c-erbB2. t...at.cc.. .tcc.gaggg cc.gtat..a .t..gcgcc. gc...gtgac .gcc.g.ccc
c-erbB3. .agt.t..ag g.gttt.tg. a.ccag.tgt cc.cat..ct ttgtggtgga .c.aacatcc
c-erbB4. g.aa..ta.. c...tg.agc attctgtgt. aagaaatgtc caca.aa.tt .gtggta.at
```

Figure 4.15: Sequence alignment data for the *ERBB* family members.
Sequence data was obtained from the ncbi website and aligned at the start codon for each sequence using Bioedit software. Gaps represented by '.'indicates base homology to the *ERBB1* sequence. ___ shows AS1 binding site, ___ shows AS2 binding site.

a)

C   LP   20   100 250 500   Dose (nM)

ERBB2 (170kDa)

Actin (27kDa)

b)
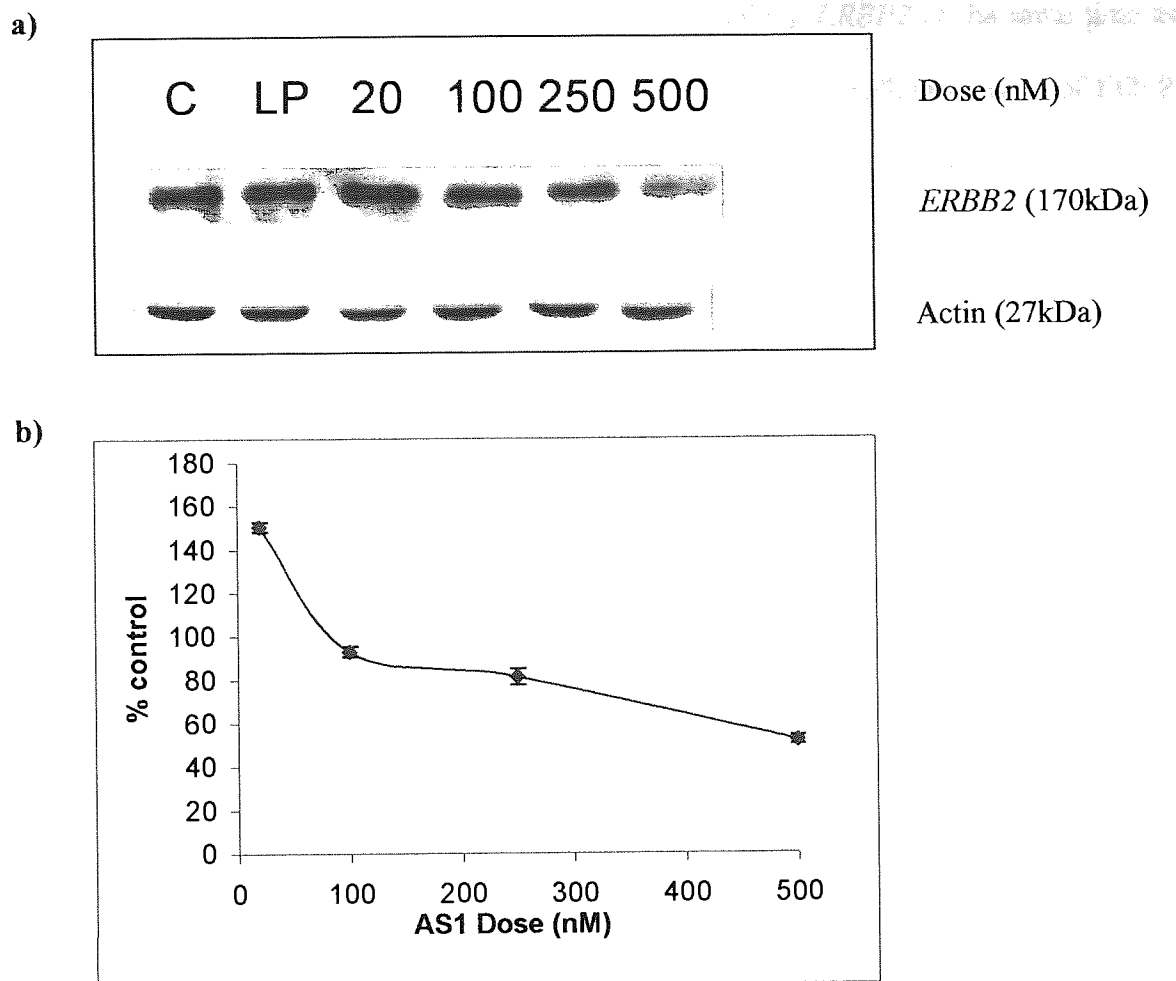
Figure 4.16: Effect of AS1 dose on ERBB2 protein expression levels over 48 hours.
A431 cells were plated in 24 well plates and cultured for 48 hours in the presence of AS1 complexed with lipid after which period protein expression levels were determined by western blot analysis. After 48 hours cells were lysed and total cell lysates (8µg per well) analysed by 7.5% SDS-PAGE and immunoblotting using either *ERBB2* or actin antibodies (section 2.9.5). **a)** Image depicts representative blot of duplicate experiments **b)** represents analysis by densitometry. C represents control untreated lysates and LP represents lipofectin^{TM} only treated cells.

As predicted by the sequence homology data, there is an effect on *ERBB2* protein expression levels (shown in figure 4.16) by AS1 but it is not as great as that found with *EGFR*. AS1 at 500nM allows a 49% (±2%) inhibition of *ERBB2* protein levels compared to that of 98% (±3%) with *EGFR*. Although this indicates some non-specificity of AS1 it is not necessarily detrimental to the objectives of the study. Within the normal cellular signalling pathway of EGFR, ERBB2 can play an important role. Upon dimerisation of the receptors, it is often found that ERBB1 and ERBB2 dimerise together to form the

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receptor complex (Sweeney *et al.*, 2001). Thus by inhibiting *ERBB2* at the same time as *ERBB1* an increase in the desired effect of inhibition of the signalling cascade of EGFR and subsequent growth inhibition can be achieved. Furthermore it is possible that there is little detrimental effect on other pathways of the cell that utilise ERBB2 signalling as the inhibitory effect is not 100%, thus other pathways that utilise ERBB2, for example phosphokinase C release and the subsequent lipid signalling pathways, will still function (Sweeney *et al.*, 2001). In order to study this further it would be necessary to look at studies concerning the other pathways of *ERBB2* and the characteristics of the cell that represent these pathways to determine the full effect of its inhibition.

4.3 Effect of AS1 on *EGFR* mRNA Levels.

So far the data presented in this chapter points to a very promising scenario for the use of AS1 as an inhibitor of *EGFR*. It is established that it not only has a sequence-specific effect on cell growth but also on EGFR protein levels. However, the question remains as to how AS1 is achieving this effect. So far the only data to suggest a mechanism of action is that of the 2' *O*-methyl derivative AS1 which showed no biologically significant effect on cell growth or protein levels indicating that the mechanism is likely to be an RNase H type mechanism as opposed to a steric hindrance effect. If this assumption is true then it is plausible that there will be a decrease in mRNA levels of the specified target, in this case *ERBB1*. This is due to the way in which the RNase H allows degradation of the ODN mRNA duplex allowing the ODN to continue functioning but the mRNA to be broken down (Crooke, 1998b).

Work performed in the laboratory by Dr Marcus Hughes had already provided *in* vitro

data that suggested that AS1 was capable of activating RNase H thus was capable of

allowing mRNA degradation within the cell of the target (Hughes, 2001). However, to

further investigate the possibility of an RNase H dependant mechanism it was necessary

to establish a decrease in the mRNA levels within the cells. There are various techniques

available to determine the mRNA levels and here two methods were used, semi-

quantitative RT-PCR (section 2.10.2) and RPA (section 2.10.3).

4.3.1 RT-PCR of ERBB1 mRNA Levels.

RT-PCR is a very sensitive technique that allows determination of any changes in the

mRNA levels within a pool of cells. The cells are treated as necessary with the antisense

and then collected before lysis and total RNA extraction. The mRNA is then subjected to

RT-PCR and electrophoresed on an agarose gel to allow quantification of the target

mRNA that has been amplified with specific primers. In order to allow comparison of the

levels of mRNA within different cell populations, actin primers were used in order to

show equal loading of mRNA throughout all samples. In this case multiplex RT-PCR was

performed allowing a direct form of comparison between the samples and thus allowing

the procedure to be semi-quantitative.
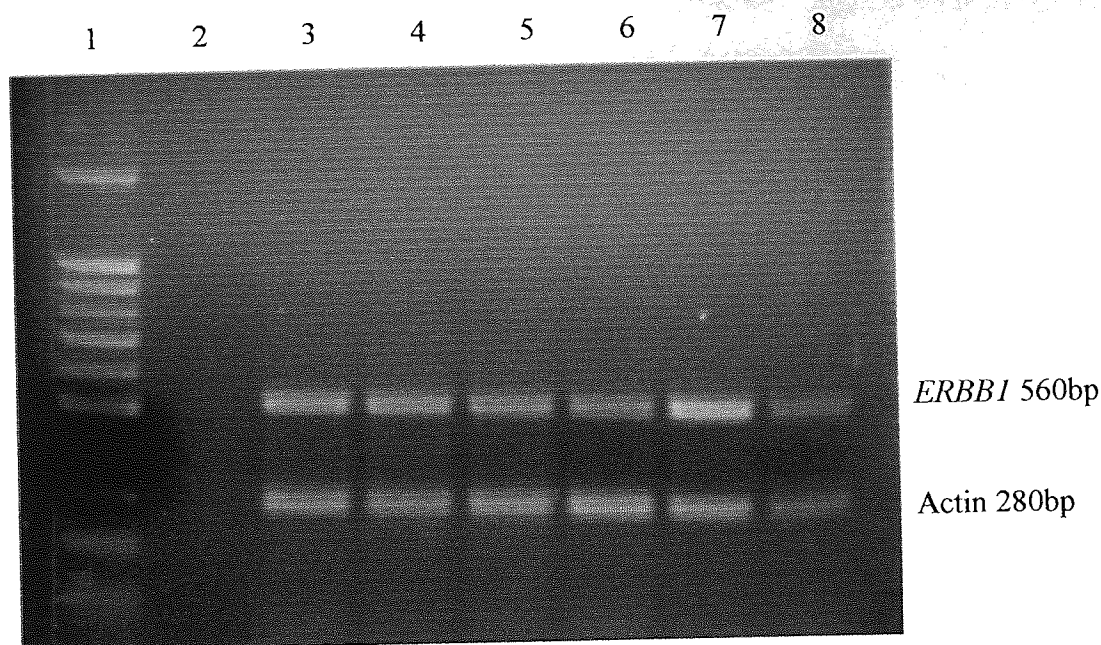
Figure 4.17: Effect of AS1 dose on ERBB1 mRNA levels in treated and control samples analysed by RT-PCR and agarose gel.
A431 cells were plated into 24 well plates and after 24 hours incubated with the relevant compounds twice at 24 hourly intervals. After 48 hours of treatment, cells were collected and total RNA extracted before RT-PCR was performed and the results analysed by agarose gel electrophoresis. Lanes represent 1=100bp ladder, 2=empty, 3=20nM AS1, 4=100nM AS1, 5= 250nM AS1, 6= 500nM AS1, 7= lipofectin only and 8= untreated control cells. Image is representative blot of triplicate experiments.

Due to the relative difficulty of achieving equal loading due to the low amount of total RNA isolated from the cells the loading of the samples is considerably different and thus it is difficult to see from this blot the effect of the AS1. Densitometric analysis of both the actin and *ERBB1* bands however provided a means to equilibrate the samples and as both primers were used at the same time, a direct comparison can be made between samples in this way.

Dose (nM)	% Control	±SD
Control	100	0
Lipofectin	125	7.6
20	95.45	5.6
100	107.345	13.2
250	91.73	5.3
500	81.2	1.8

Table 4.2: Densitometric analysis of RT-PCR of AS1 treated A431 cells over 48 hours from figure 4.17.
NIH imaging software was use to analyse the RT-PCR figures represented by the gel in figure 4.17 and ratio analysis performed between the actin levels and the *ERBB1* levels. The control untreated lane was taken as 100% mRNA level and other samples compared to this.

From the RT-PCR results it does not appear that there is a biologically significant difference (p>0.05) between the control / lipid treated cells and the AS1 treated cells as regards to mRNA expression levels. Although at 500nM there is about a 20% decrease in mRNA levels and that this is statistically significant it can be seen from the cell growth assays that 20% inhibition of cellular growth can be observed with the lipid alone. However, it may be that due to the small amount of mRNA required to allow synthesis of vast amounts of protein a decrease of 20% in the pool level of *ERBB1* is sufficient to elicit the response found on protein levels and cellular growth.

In order to clarify whether the effect found with the RT-PCR was true and that it was repeatable with other methods northern blotting was performed, However, it was soon realised that due to the low cell numbers involved the amount of mRNA available when probed was not enough to allow a clear picture using this method. Thus an alternative method was employed, that of the ribonuclease protection assay (RPA).

4.3.2 RPA Analysis of ERBB1 mRNA Levels.

RPA is a technique that is often used as a replacement to northern blotting and it offers many distinct advantages over the blotting procedures. It is much more sensitive and relatively quick but most importantly it offers the ability to probe for multiple mRNA targets at a time as opposed to northerns where the membrane would have to be stripped and re-probed for different species. Thus like the RT-PCR used it is semi-quantitative. Perhaps the biggest draw backs to the technique is that it requires radiolabeled probes to be made fresh each time as they degrade rapidly and thus the procedure can take a long time if major optimisation is required.
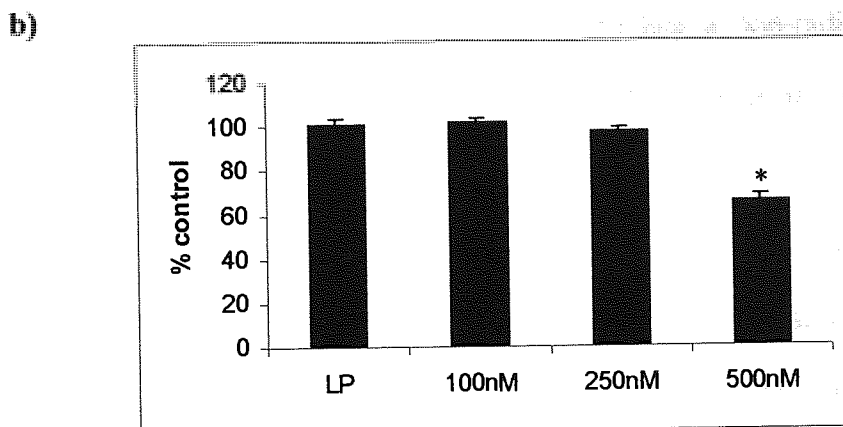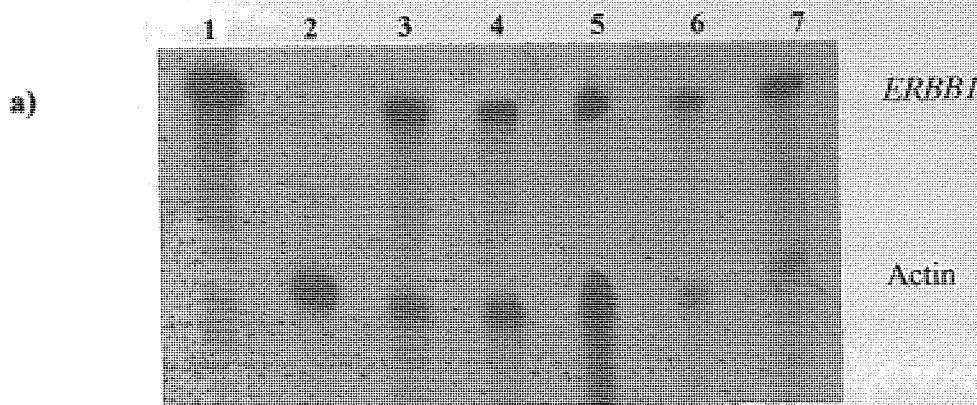
Figure 4.18: Representative autoradiograph of RPA analysis of AS1 treated A431 cells over 48 hours with two doses and graphical representation of densitometric analysis of mRNA bands.
a) As for the RT-PCR cells were treated with the required compounds for 48 hours at 24 hourly intervals before collection and RPA analysis. Samples of the two probes used were also run on the denaturing gel for easy identification of bands. Lanes 1= *ERBB1* probe, 2= actin probe, 3= 100nM AS1, 4=250nM AS1, 5= 500nM AS1, 6= Lipofectin treated and 7= untreated control cells. b) Graphical representation of the RPA gel after densitometric analysis. Control untreated cells were assumed to be 100% and effect of treatments compared to this. Blot is representative to duplicate experiments. * represents significant difference (p<0.05) to the control.

Although the image obtained (figure 4.18) does not clearly show the effect of AS1 dose the representational densitometric analysis clearly indicates that there is a significant knockdown in the mRNA levels of *ERBB1* (67.6% ±0.8% of control). The RPA obviously requires further optimisation to give a clearer picture of this effect with the main problem being the equal loading of samples. In this figure it is clearly shown that there is a lot more 500nM sample loaded (indicated by the darkness of the band for the actin) and thus it is not clear that the *ERBB1* band for this sample is less in proportion to

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the other doses. Also the lipofectin only sample is very low but equally so for both genes thus it is still fairly equal with little reduction in the *ERBB1* level (101% ±3.1%). Given time it is evident that a clearer picture of the knockdown of *ERBB1* mRNA by AS1 can be obtained with further optimisation of this technique and that although some mRNA reduction was found with the RT-PCR analysis this technique is much more sensitive at detecting small changes in mRNA levels. As this assay has now become popular, it has been further developed by manufacturers enabling a non-radioactive equivalent to be developed. This chemiluminescent method will speed up the whole process further as target probes will not degrade and it will enable a much clearer picture to be obtained.

It is difficult to compare these results with others in the antisense field due to the lack of published work showing cell growth assay, protein reduction and mRNA effects in similar treatments. Chiang *et al.*, (1991) showed a 70% decrease in mRNA levels compared to control cells when investigating the effect of *ICAM1* expression inhibition by antisense ODNs using northern blotting. Whereas, Veal *et al.*, (1998) showed complete inhibition of *GAG* mRNA levels in HIV-1 cells with 10μM antisense ODN. Although these published effects are greater than those found here it is difficult to make a direct comparison due to varying concentration of ODN and dose regime employed as well as cell type and gene of interest.

4.4 Mode of Cell Death in Antisense Treated Cells

It is evident from the results obtained with the proliferation assay, western analysis and mRNA analysis that the DNA chip designed AS1 and to some extent AS2, are capable of exerting an antisense effect on A431 cells. As the end point of the antisense treatment is

not only cell growth inhibition but cell death it is important to determine the mechanism of how the cells are dying. There are two main ways by which cells can die, necrosis and apoptosis (programmed cell death).

Necrosis, also called accidental cell death or pathological cell death, is a passive process that occurs when the cells are exposed to extreme physiological or environmental stresses, for example, due to lack of oxygen, mechanical damage or an exposure of cells to toxic chemicals (Voet & Voet, 1995). Following necrosis cells lose their ability to maintain homeostasis, which leads to cellular oedema, release of intracellular ions and eventual osmotic lysis of cells. In necrotic death the cells respond to pathological changes initiated outside of the cell elicited by a large number of changes that trigger a change in the membrane permeability of the cell. In comparison in the process of apoptosis the cells participate actively in the cell death mechanism. Apoptosis is characterised by cellular and more importantly, nuclear shrinkage with chromosomal condensation (Al Rubeai & Singh, 1998). This causes DNA fragmentation and blebbing of the cellular membrane. Apoptotic cells are normally mopped up by macrophages within the body or in culture appear as apoptotic bodies and debris in the media. Apoptosis is a major pathway in most if not all cells of the body that enables cellular maintenance, e.g. monitoring of DNA damage to be dealt with effectively (Lodish *et al.*, 1995). Upon damage to a cell the apoptotic pathway can be initiated to allow cell removal and the prevention of further aberrant cells being produced. It is a major pathway that is targeted in the process of cancer cell development and one by which tumourigenic cells can overcome cell cycle regulation.

There are many methods now available in which to determine the presence of apoptosis in a dying population. Some of these are based on the morphological changes occurring, for example, blebbing of the membrane, cell shrinkage and nuclear condensation. Other methods are based on the presence of the DNA fragmentation as an indication of apoptosis.

In this study it was evident that cell death was occurring by the inhibition of EGFR protein synthesis thus leading to a decrease in active growth factor signalling pathways. Thus fluorescence-associated cell sorting (FACS) analysis was used to determine the presence of DNA fragmentation within the samples. Cells were analysed for cell cycle distribution with control cells being sorted into the main phases of the cell cycle dependant on the DNA number per cell present. Cells in G1 have single copies of the DNA whereas cells in G2/M phase have two sets of chromosomes due to the fact that they are about to divide into two cells. Two peaks on the cell distribution diagram represent these populations. The phase linking these two peaks is the S phase which will contain a mixture of cells that are about to divide and thus have either one or two sets of DNA (figure 4.19). Cells that are undergoing apoptosis tend to be grouped in the area before the G1 peak and are termed the sub-G1 proportion of cells.
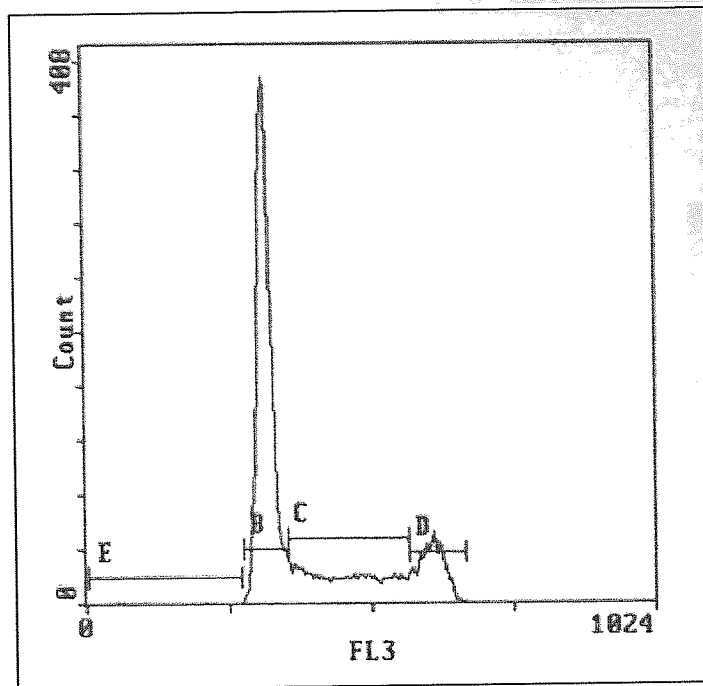
Figure 4.19: Representative plot of cell cycle distribution of control cells.
Cells are divided into phase's dependent on the amount of DNA detected by FACS that has been stained using propidium iodide a DNA intercalating dye. Gate B = G1 phase, Gate D = G2/M phase and Gate C = S phase. Gate E is representative of a sub-G1 peak.

Cells treated with various doses of AS1 were collected, along with the apical media, and centrifuged before fixing and staining with a DNA intercalating dye, propidium iodide. Cells were then analysed by FACS for the presence of DNA fragmentation represented by a sub-G1 peak. The distributions of cells between areas of cell cycle were expressed as a percentage of the total cells and the percent of cells in sub-G1 displayed in figure 4.20 to determine the effect of AS1 on apoptosis levels.
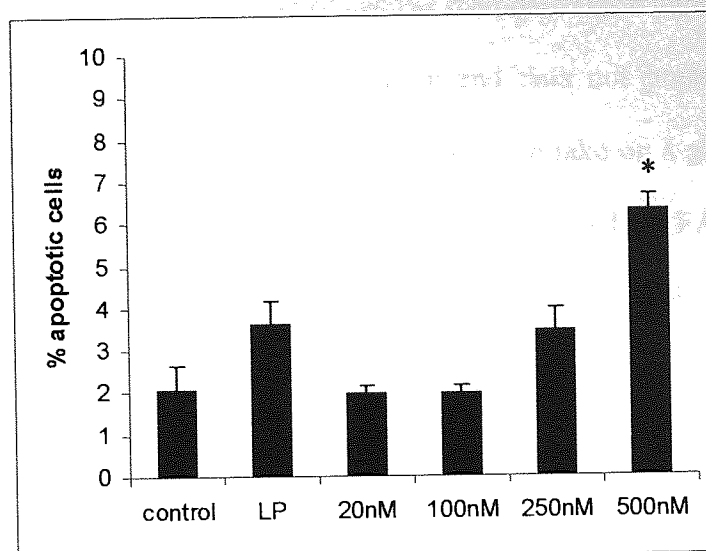
Figure 4.20: Effect of AS1 of apoptosis levels determined by the sub-G1 peak produced using FACS analysis.
Cells were treated with AS1 and lipofectin as two doses over 48 hours before cells were collected by centrifugation, fixed and incubated with an intercalating dye overnight prior to cell cycle analysis by FACS. LP = lipofectin treated cells. Data is representative of triplicate experiments. * represents a significant difference ($p<0.05$) to that of the control.

From the apoptosis studies above it can be seen that at 500nM AS1 there is significantly ($p<0.05$) more apoptosis than that found in the control cells. However it is deemed that this effect is still not that great as an overall percentage of apoptosis. It was expected that due to the extreme effects on cell growth caused by AS1 a more substantial effect on apoptosis levels would be observed. The distribution of the cells throughout the remaining phases of the cell cycle was fairly consistent independent of the treatment received and thus the cells were not being arrested in a particular phase of the cell cycle instead.

An explanation for the lack of significant effect on apoptosis seen could be due to the method by which the sample is analysed or due to the fact that the cells are not dying but simply losing their adherent properties. It was noticed that upon treatment of the cells with AS1 the cells lost their adherent properties and thus displaced into the apical media. Although this media was collected when collecting samples it is evident that the cells if

dead would be lighter due to loss of cellular material. These lighter cells are thought to be less likely to be pelleted by centrifugation and thus not present for analysis of DNA content by FACS. One such theory is that cells can take on a ghost-like structure, which have no DNA present and thus will not be represented by FACS analysis (Tey *et al.*, 2000). In order to clarify whether the lack of apoptosis is a function of the method used or whether the cell number loss observed in the previous assays is due to loss of adherent properties, needs to be studied further.

4.5 Conclusions

In this chapter the biological efficacy of various oligonucleotides directed against the *EGFR* mRNA were investigated according to their effect on cell proliferation, protein levels and mRNA levels.

Two antisense sequences, targeted to *EGFR*, were selected in chapter three and were thus synthesised as phosphorothioate and 2'*O*-methyl derivatives as well as a chimeric form. The ODNs were tested for efficacy against the A431 cell line; a vulval carcinoma cell line that greatly over-expresses EGFR levels at 10-50 fold higher than seen in other cell lines (Ullrich *et al.*, 1984).

Results show that the sequences AS1 and AS2 have a dose dependant effect on the growth of A431 cells over time. The control ODNs tested covered a wide range but none of them elicited a significant effect on cell growth inhibition. These included a novel ODN sequence (AS3) selected using the DNA chip design technology, which enabled further proof that the technology could not only potentially select active sequences but also provide inactive sequences. Cell studies were confirmed as showing a true antisense

effect by the encouraging reduction in protein levels of EGFR with AS1 treatment, although the effect required a double dose of AS1 over 48 hours. The reason why two doses of AS1 were needed was thought to be due to the sequestered levels of EGFR already present within the cell. These levels of EGFR are thought to mask the effect found with AS1 and thus time was needed for these levels to be degraded before the effect of AS1 could be evident. This theory can be tested with the use of fluorescent-labelled EGFR antibody. This would bind to the EGFR protein within the cell and thus provide a localisation picture of the distribution of the EGFR within the cell. After 24 hours treatment it is expected that the distribution would be throughout the cell with EGFR being present at the membrane and in the cytoplasm where the circulating pools are still present. After AS1 repeated doses the levels of EGFR should be much less with no or little representation at the membrane so that it cannot accept the growth factors from the medium thus cell growth is inhibited. Further western analysis showed that AS1 also had a significant knockdown effect on ERBB2 levels, another member of the EGFR family, but the effect was not as great as that found with EGFR and was somewhat expected due to the high sequence homology at the ODN binding sites between the two sequences.

AS1 effect on mRNA levels was found although not as great as those found in other studies. However, without studies performed on similar cell lines or targets it is difficult to make a direct comparison of the results found. From these results and those found using 2' O-methyl derivative, it was postulated that the AS1 was acting via an RNase H type mechanism. Finally the mode of cell death observed was investigated to determine whether it was by necrosis or apoptosis. FACS analysis showed a dose dependant effect on apoptosis levels of AS1 with a significant increase in apoptosis levels with 500nM

AS1 although further investigation using other techniques is required to clarify whether

apoptosis is the main event responsible for cell death found with AS1 treatment.

Chapter 5.0: Future therapy for chip designed ODNs including combination treatments with conventional drugs and other ODNs.

Due to the vast heterogeneity of some tumours, especially gliomas, it is unlikely that a single treatment regime will be sufficient to reduce the tumour size and reverse the malignant phenotype. It is with this in mind that the issue of combination therapies is addressed here. We have examined the effect of combinations of dual antisense molecules on A431 cell growth and antisense ODNs combined with conventional chemotherapeutic drugs. We are not making the statement that the combinations used here are the best methods of addressing glioma therapy but are merely exploring the possibilities of the types of combinations available and the problems that may arise in these treatments. It is evident from our findings that for a combination therapy to be put into the clinic it will require not only feasibility of the treatment alone but also specified treatment regimes, including optimisation of aspects such as delivery, to be carefully considered.

As well as the effect of combination treatments, the problem is raised as to the use of an accurate *in vivo* model for gliomas to test antisense compounds. Although traditionally xenograft models have been utilised, they do not often represent an accurate model for glioma therapy in humans. To establish the effect of AS1 and AS2 effects in a glioma model, primary cell lines have been utilised to determine the efficacy of antisense treatment.

5.1 Evaluation of DNA Chip Designed ODNs in Primary Brain Tumour Lines

From the results obtained so far concerning efficacy of DNA chip designed ODNs to the *EGFR* mRNA, great promise for the use of these ODNs as potential therapeutic agents has been shown. However, all of the work shown so far has utilised these ODNs *in vitro* on an established epidermoid carcinoma cell line, A431, which over-expresses the target gene. The next step in any assessment of a potential therapy is to determine the effects of the potential drug *in vivo*. This usually is investigated with the use of animal models, either mice or rats, in which a tumour (or xenograft model) is developed within the animal, in this case situated in the brain, and the therapy is injected into the tumour site for monitoring of the efficacy of the therapy within the target site/ organ. In order to determine the effect of our ODNs for the potential therapy of glioblastoma multiforme it is essential to have a tumour model that represents the genetic profile and epidemiology of this cancer in humans. The use of accurate animal models are powerful tools in the investigation of the development of glioma tumours biology and the greater understanding of these tumours can only aid in the development of new therapies and strategies to fight this disease. There are many methods for developing gliomas *in vivo* but the most widely used in preclinical trials to date is xenograft models (Palma *et al.*, 2000; Kondo *et al.*, 1998, Khachigian *et al.*, 1995). Usually a cell line derived from a human or animal glioblastoma would be cultured *in vitro*, which could then be injected into an animal model to form a full glioblastoma tumour.

The histology of xenografts created from gliomas is however usually dissimilar to that found in human tumours and the genetics of the resulting tumour is usually unknown.

Xenograft tumours are developed in immunodeficient mice so that their immune defence mechanisms do not attack the tumour cells upon injection. This however means that once the tumour is developed the mice do not show any immunological side effects to either the tumour or the therapy thus do not provide a good model for humans (Dai & Holland, 2001). In spite of these limitations of xenograft models they have provided reproducible tumours and are thus still the major method in therapy testing *in vivo*. Their use as predictors of human response to a therapy however has not provided good correlation and is thus not an ideal system.

Due to the factors outlined above and the lack of facilities to perform animal testing of the ODNs developed it was decided to firstly determine the effect of the ODNs developed on primary cell lines. These cell lines however at the time were not characterised fully for their genetic epidemiology and thus it is uncertain as to whether they over-express EGFR and thus whether the ODNs could elicit a response. The cell lines were developed from human brain tumours and were initially obtained as biopsy samples. Upon receiving the sample of tissue, a cell line was developed that could be maintained in culture for a short period of time, so that testing of the antisense compounds could be performed. The cell lines were developed at the Department of Neurological Surgery, The Institute of Neurology, London and kindly donated for the testing of our antisense compounds. These cell lines are not established cell lines and are thus only capable of cell division *in vitro* up to a limited number of passages. After this time the morphology of the cells begins to alter and is thus no longer a reliable model to study for gliomas.

Two of the cell lines established from tumours (IN859 and IN2045) were used to study the effect of AS1 and AS2 ODNs on cellular proliferation. These studies were performed by exactly the same method as that used on the established cell line, A431.

IN859 cells were established from a biopsy sample taken from a patient suffering from an uncharacterised brain tumour. Cells were treated with AS1, AS2 and control ODNs to determine the effect of ODN mediated *EGFR* knockdown on cellular proliferation.

Figure 5.1: Effect of AS1 on IN859 cell growth over time.
AS1 ODN was complexed with lipofectin^{TM} (LP) and added to IN859 primary cells in the presence of serum-free media in either 24 well plates or 96 well plates for 4 hours followed by an incubation in fresh complete media for a further 20 hours at 37°C and assayed for cell growth as described in section 2.8.1.2 by crystal violet assay and confirmed by trypan blue exclusion (2.8.1.1). All treatments were in the presence of 7.5µM lipofectin^{TM} with the cell number being expressed as percentage control of untreated cells. Data represents the mean n=4±SD. * represents a significant difference (p<0.05) from the control.
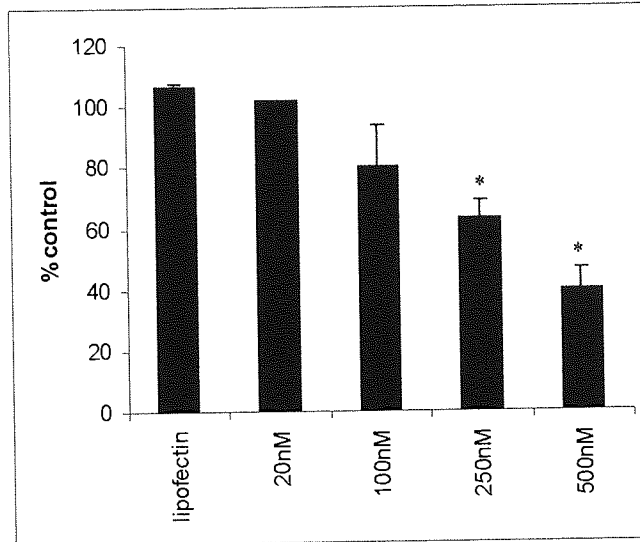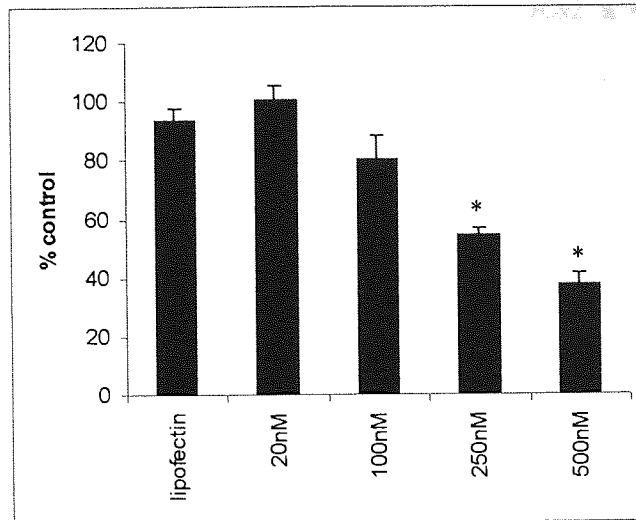
Figure 5.2: Effect of AS2 on IN859 cell growth over time.
AS2 ODN was complexed with lipofectin^{TM} (LP) and added to IN859 primary cells in the presence of serum-free media in either 24 well plates or 96 well plates for 4 hours followed by an incubation in fresh complete media for a further 20 hours at 37°C and assayed for cell growth as described in section 2.8.1.2 by crystal violet assay and confirmed by trypan blue exclusion (2.8.1.1). All treatments were in the presence of 7.5μM lipofectin^{TM} with the cell number being expressed as percentage control of untreated cells. Data represents the mean n=4±SD. * represents a significant difference (p<0.05) from the control.

The above results show that increasing doses of AS1 and AS2 give a decrease in cell number compared to that of the untreated control cells. Cells treated with lipofectin^{TM} alone did not show any decrease in cell number indicating that the effects observed with the ODNs is not simply due to toxicity of the lipid. This is important to clarify here as no optimisation of delivery has been performed. As stated in section 3.3, optimal delivery of ODN to the cells is dependant on many factors including cell type (Bennett, 1998). Furthermore as these cells are not an established cell line they are a lot more sensitive to changes in their environment thus the lipid concentration used here, to enable direct comparison with A431 cell responses, may have been toxic to the primary cells. As can be seen from the results the lipid concentration of 7.5μM was not toxic, as it had no effect on the cell viability as compared to the untreated control cells. There is also a dose response to the ODN evident with a 500nM dose of AS1 eliciting a 60% (±7%) decrease

in cell number compared to control cells and AS2 a 63% decrease (±4.2%). These

decreases in cell number are significant (p<0.05) and indicate that the ODNs against

EGFR are showing an inhibitory effect by some means to the cell. The nature of the

effect is difficult to determine from these experiments alone. It could be due to a toxic

response to the ODNs as again these cells are quite sensitive and thus could simply be

sensitive to the ODN chemistry. Also the effect seen could be a non-antisense effect in

the respect that the ODNs could simply be binding to proteins within the cell. Both of

these scenarios can be investigated with the use of control ODNs.
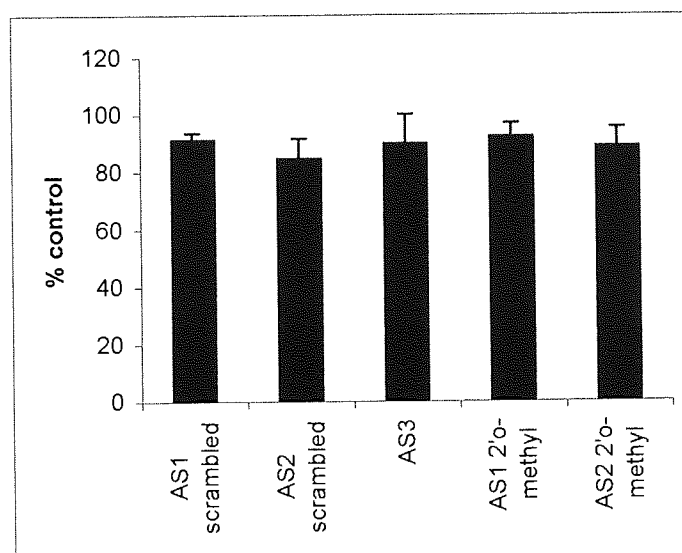
Figure 5.3: Effect of controls on IN859 cell growth over time.
Control ODNs were complexed with lipofectin[TM] (LP) and added to IN859 primary cells in the
presence of serum-free media in either 24 well plates or 96 well plates for 4 hours followed by an
incubation in fresh complete media for a further 20 hours at 37°C and assayed for cell growth as
described in section 2.8.1.2 by crystal violet assay and confirmed by trypan blue exclusion
(2.8.1.1). All treatments were in the presence of 7.5μM lipofectin[TM] with the cell number being
expressed as percentage control of untreated cells. Data represents the mean n=3±SD.

As can be seen in figure 5.3 no significant effect (p>0.05) was observed with any of the

controls used indicating that a sequence specific antisense effect might be occurring due

to the ODNs targeted against *EGFR*. Although this cannot be completely confirmed by

the lack of effect found with the controls it is an encouraging find. The effect of AS1 and

AS2 on protein levels and mRNA levels of the *EGFR* would be a step towards confirming an antisense-specific knockdown of the target by the ODNs in this primary cell line as was found with the A431 cells in chapter 4. The only difference between the treatments of the two different cell lines is that the primary cell line was returned to complete media following the initial 4-hour treatment, as the cells did not survive when grown in serum-free media for prolonged periods.

Unfortunately when western blotting was performed on primary samples no signal for EGFR could be determined. This indicates that the levels of EGFR even in the control samples are relatively low and beyond the detection limits of the assay. It would appear therefore that although AS1 and AS2 are having an effect on the growth of IN859 cells it cannot be established whether this is a specific effect as the protein levels cannot be studied. However, when performing a similar analysis on an established glioma cell line, U87-MG, EGFR protein levels could not be determined either. The phenomena of EGFR amplification and rearrangement are less commonly observed in cultured glioma cell lines than in solid tumours (Filmus *et al.*, 1985). In addition, cell lines derived from primary tumours often lose any mutated alleles when maintained in cell culture (Han *et al.*, 1996). It appears that as A431 cells greatly over-express EGFR protein, it is easily detected by western blotting analysis, but glioma lines although they may have large amounts of EGFR compared to other cell lines still do not express large enough levels to be readily detected by standard western blotting techniques. It is proposed that this cell line should not only be analysed for its genetic alterations but should also be subjected to immunoprecipitation prior to western blotting so that the levels of EGFR even if not over-expressed can be analysed. Only in this way can we be more confident that the effect observed here by AS1 and AS2 is a true antisense mechanism within these cells (Stein, 1999).

In comparison to the effects found within A431 cells however, the effect observed on cell

number in both cases is remarkably similar. They both exhibited approximately 60%

inhibition of cell number compared to untreated controls with the control ODN sequences

having little or no effect.

5.1.2 Effect of ODN Treatment of IN2045 Cells

The second primary cell line studied was that of IN2045. This was also resected from a

patient at biopsy and established as a growing cell line. Again the cell line had a finite

lifetime before it began to transform and thus experiments were performed at low

passages. Cells were treated with AS1, AS2 and control ODNs as for the cell lines A431

and IN859.

Figure 5.4: Effect of AS1 on IN2045 cell growth over time.
AS1 was complexed with lipofectin™ (LP) and added to IN2045 primary cells in the presence of serum-free media in either 24 well plates or 96 well plates for 4 hours followed by an incubation in fresh complete media for a further 20 hours at 37°C and assayed for cell growth as described in section 2.8.1.2 by crystal violet assay and confirmed by trypan blue exclusion (2.8.1.1). All treatments were in the presence of 7.5µM lipofectin™ with the cell number being expressed as percentage control of untreated cells. Data represents the mean n=3±SD. * represents a significant difference (p<0.05) from the control.
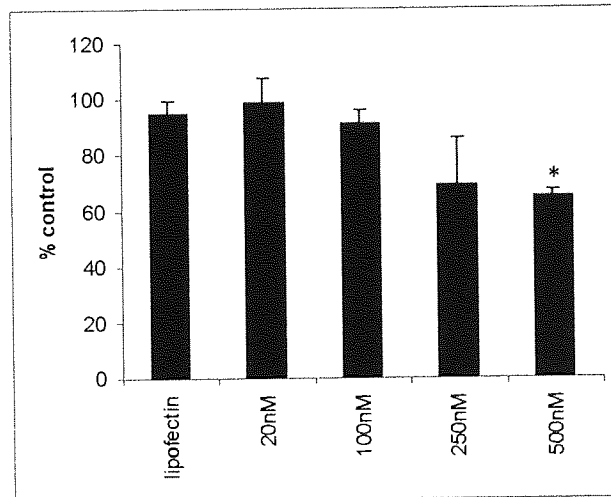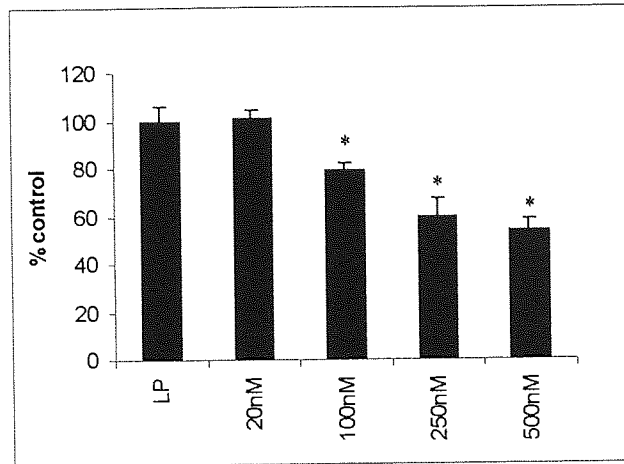
Figure 5.5: Effect of AS2 on IN2045 cell growth over time.
AS2 was complexed with lipofectin™ (LP) and added to IN2045 primary cells in the presence of serum-free media in either 24 well plates or 96 well plates for 4 hours followed by an incubation in fresh complete media for a further 20 hours at 37°C and assayed for cell growth as described in section 2.8.1.2 by crystal violet assay and confirmed by trypan blue exclusion (2.8.1.1). All treatments were in the presence of 7.5μM lipofectin™ with the cell number being expressed as percentage control of untreated cells. Data represents the mean n=3±SD. * represents a significant difference (p<0.05) from the control.

Again as with the antisense treatment of IN859 cells, the lipid alone had no biological effect on cell number compared to the control cells (5%±4%) thus indicating that at the 7.5μM concentration used it was not in any way toxic to the cells proliferation status. The effects of AS1 and AS2 however were observed. In the case of AS2 there was a significant (p<0.05) dose response as found with A431 and IN859 cells but with AS1 although there was only some significant effect (p<0.05) at 500nM (35.2% ±2.8%) there is no real dose response observed. It appears that these cells are less sensitive to the effect of antisense treatment towards EGFR and would initially point to the scenario that the mutation allowed in these cells that allows them to become tumorigenic is not one of EGFR amplification. However, due to the fact that AS2 seems to be having some effect (46% ±5.2% inhibition) it was deemed necessary to look at the control sequences. It could be that in these cells the AS2 is having an effect through a non-antisense mechanism in that there are motifs within this ODN that the IN2045 cell line is

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susceptible to as far as non-specific binding is concerned (Matveeva *et al.*, 2000). Alternatively it could be that the folding of the mRNA within this cell line is slightly different in that it allows AS2 to bind efficiently but some change in the secondary structure does not allow AS1 to bind with the same intensity.
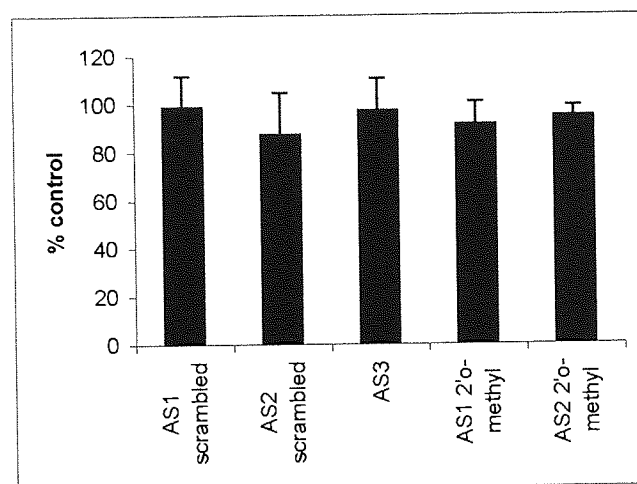
Figure 5.6: Effect of controls on IN2045 cell growth over time.
Control ODNs were complexed with lipofectin^{TM} (LP) and added to IN2045 primary cells in the presence of serum-free media in either 24 well plates or 96 well plates for 4 hours followed by an incubation in fresh complete media for a further 20 hours at 37°C and assayed for cell growth as described in section 2.8.1.2 by crystal violet assay and confirmed by trypan blue exclusion (2.8.1.1). All treatments were in the presence of 7.5µM lipofectin^{TM} with the cell number being expressed as percentage control of untreated cells. Data represents the mean n=3±SD.

As can be seen in figure 5.6 none of the controls used either for AS1 or AS2 had any significant effect ($p<0.05$) on the cell number when compared to the control cells. This would indicate that the effect observed with AS1 and more so with AS2 is likely to be an antisense effect and not a non-specific effect as thought. Thus it is postulated that there is some difference between the two cell lines, IN859 and IN2045, that allows for this variation in treatment. The reduced effect overall compared to that in IN859 cells could be due to the level of EGFR expression present and as previously stated this should be investigated along with the entire genetic alterations present within these cells to determine the mode of tumorigenesis. It is proposed that the main explanation for the variation observed however is due to the vast heterogeneity found in most gliomas

(Shapiro & Shapiro, 1998). It is because of this heterogeneity that treatment of gliomas is difficult; a treatment that works for one patient may not work for another. As is shown here the same antisense molecule that works well in A431 and IN859 cells does not work so well in another tumour, IN2045. Although the treatment does not show any adverse effects *in vitro* thus would potentially not harm a patient if administered, it may be that a more effective treatment regime could be found for that patient. It is with this in mind that the idea of combination therapy appeals.

5.2 Potential Therapy Regimes for the Treatment of Gliomas.

Combination therapy is already prevalent with standard drugs for the treatment of cancer in the clinic to date, with the combination of radiotherapy and chemotherapy being a common treatment regime (Fine *et al.*, 1993) along with surgery where plausible. It was this indication that led to the decision to investigate some potential combination treatments with our antisense ODNs together with other ODNs that showed promise as potential antisense drugs and with more standard cytotoxic drugs. Conventional drugs, such as temozolomide provide some beneficial effects to patient survival but are not substantial and are often accompanied by adverse side effects, including myelosuppression (Newlands *et al.*, 1992). Combination therapy could provide the advantage of temozolomide, which has been shown to cross the blood brain barrier in mice (reviewed in Friedman *et al.*, 2000), along with the specificity of antisense treatment.

The selection of the type of combination therapies to investigate was simply due to personal choice and the aspect of availability and it is not expected that they are likely to be the most optimum combination therapies to use for gliomas or any other type of

cancer. However, the choices were made dependant on whether these potential therapies had shown good effects so far either as a cytotoxic drug or as an antisense drug *in vitro* and thus the likelihood of success was greater. It is likely that the type of combination therapy used would have to be custom designed to a particular patient or tumour type.

5.2.1 Antisense to Antisense Combinations.

Perhaps the most obvious combination to consider first would be the combination of two antisense molecules to a target to determine whether this would have a greater effect than any single molecule alone. This can be achieved with two ODNs targeting the same gene or two different genes. In addition the two ODNs can either target completely different pathways within a cell, if there are two distinct mechanisms of cellular transformation involved, or two genes on the same pathway but acting at different stages.

a) AS1 and AS2 Combination

The simplest method by which to test combination therapy is to treat A431 cells with both AS1 and AS2 as a combination and to determine whether this regime would provide an enhanced effect to that already seen. A431 cells were used throughout the combination therapies as they have been thoroughly investigated in chapter 4. Cells were treated with AS1 and AS2 at the same time with a constant concentration of lipofectin. Three doses for treatment were chosen, 100nM, 250nM and 500nM. Although 500nM had previously shown to have the greatest effect for both AS1 and AS2 (figure 4.1 and 4.6) it was thought that a combined effect may not be obvious with such a dose as each ODN may already be working at its maximum efficiency for the cell line. As the lipid concentration

could not feasibly be increased to more than 7.5µM without cytotoxicity being initiated

(figure 3.12) this was kept constant thus both ODNs were mixed with half of the total

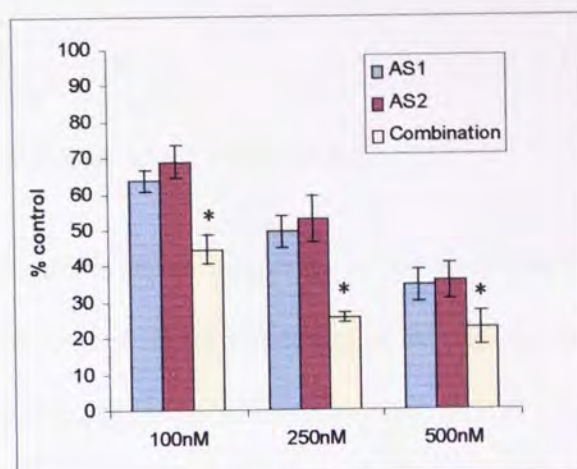lipid concentration prior to combination to allow complex formation to occur.

Figure 5.7: Combination effect of AS1 and AS2 on A431 cell growth over time.
AS1 and AS2 were mixed with a total of 7.5µM lipofectin[TM] and incubated for 5 minutes to allow complex formation prior to mixing together and addition to A431 cells. Combined ODN treatment was performed on A431 cells in either 24 or 96 well plates over 24 hours and cell number analysed as in section 2.8.1.2 by crystal violet assay and confirmed by trypan blue exclusion (2.8.1.1). Triplicate experiments were performed and error bars represent n=3 mean ±SD. * Represents significant difference (p<0.05) to the single treatment cells.

AS1 and AS2 administered to A431 cells in combination caused a significantly greater

reduction in cell number compared to individual treatments with the ODNs (figure 5.7).

Although the effect observed does not produce a super-additive effect as observed in

some combination therapies (Tortora *et al.*, 1997) it does provide an enhanced effect at a

particular dose compared to a single treatment. It is traditionally of considerable

importance to reduce any dose of drug to the minimum possible concentration in order to

still observe a significant effect with minimal or no side effects. Thus it is encouraging

that a smaller dose of ODN is required when using combination treatment to get a similar

effect to that found at a higher dose when a single ODN is employed. For example AS1

alone at 250nM allows a 50% decrease in cell number and AS2 a 45% decrease whereas

both AS1 and AS2 combined at a dose of 250nM total concentration provided a 75% decrease in cell number compared to control cells. A similar response was observed for the 500nM doses although the combined effect was not as enhanced as with the 250nM dose.

b) *C-RAF-1* and AS1 Combination

One of the downstream molecules in the signalling cascade of EGFR is that of *C-RAF-1* kinase and as such down regulation of this target could also potentially allow cell growth inhibition of cell lines over-expressing EGFR. *C-RAF-1* has been identified in the development and maintenance of certain human malignancies thus provides a good target for antisense directed therapy. Furthermore, *C-RAF-1* mutations have been shown to transform cells *in vitro* (Heidecker *et al.*, 1990) and have been associated with certain human tumours (Shimizu *et al.*, 1985).

Recent work by Monia *et al.*, (1996) has used phosphorothioate antisense ODNs targeted to *C-RAF-1* kinase to specifically inhibit the *C-RAF-1* gene expression in cell culture and *in vivo* using tumour xenograft models. The work carried out used a selection of 34 ODNs targeted to various regions of the *C-RAF-1* mRNA molecule, which were delivered to lung carcinoma cells using lipofectin™. Due to the similarities in delivery mechanism and doses used to that of our studies, the antisense ODN found to be the most active in this study was synthesised and tested for efficacy on A431 cells. The sequence synthesised and studied for its effect on A431 cell proliferation is detailed below.

C-RAF-1 kinase antisense ODN sequence (20mer)

5' TCC CGC CTG TGA CAT GCA TT 3'

Treatment with A431 cells first had to be carried out with the C-RAF-1 ODN alone so that a comparison could be made following combination studies. In the initial study by Monia et al., (1996) the cells were treated with increasing doses from 25nM to 500nM for 1-5 days in the presence of lipofectin prior to analysis of cell number. Thus similar experiments were performed here using A431 cells.

Figure 5.8: Effect of C-RAF-1 antisense ODN on A431 cell number over 24 hours.
A431 cells were treated with increasing doses of C-RAF-1 antisense ODN over time complexed with 7.5µM lipofectin. ODN treatment was performed on A431 cells in either 24 or 96 well plates over 24 hours and cell number analysed as in section 2.8.1.2 by crystal violet assay and confirmed by trypan blue exclusion (2.8.1.1). Triplicate experiments were performed and error bars represent n=3 mean ±SD. * Represents significant difference (p<0.05) to control cells.

Upon performing cell counts following one day of antisense treatment the reduction in cell number compared to that of the control was significant (p<0.05). In the A431 cells the 500nM dose decreased cell number to 82% (±4.5%) after only 24 hours of treatment. In contrast Monia et al., (1996), found that A549 lung carcinoma cells were inhibited by approximately 80% by day 3 following treatment of 500nM antisense. It can be assumed from this that C-RAF-1 targeted antisense treatment would be a valuable target for antisense treatment in cell lines that over-express EGFR. However although these results are promising they by no means show that the effect observed with the C-RAF-1 antisense ODN was sequence specific and as discussed throughout this thesis many more

studies must be performed here to determine a true antisense effect. Despite these misgivings, a dose response was observed in these cells as well as the specific activity previously (Monia *et al.*, 1996) and thus it was decided that a combination therapy utilising both *C-RAF-1* and *EGFR* antisense therapies could be potentially useful.

As with combination treatment of AS1 and AS2 the overall concentration of lipid could not be further increased thus each ODN was complexed with half of the concentration of lipid prior to mixing the two antisense molecules together. In this case AS1 and *C-RAF-1* were combined as a treatment with A431 cells being treated for 24 hours at doses of 100nM, 250nM and 500nM AS1 with the concentration of *C-RAF-1* remaining constant at 250nM to allow for comparison of effect.
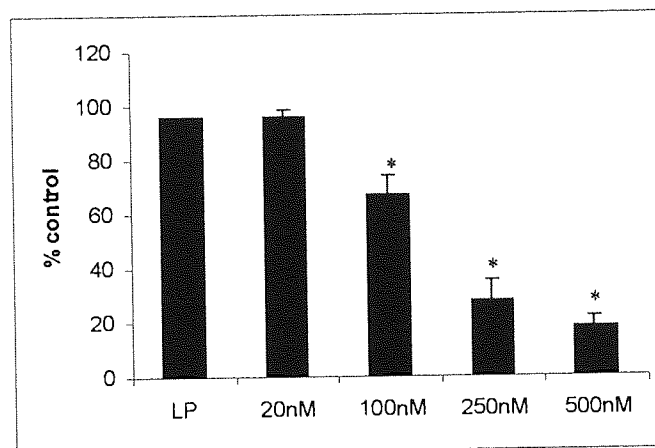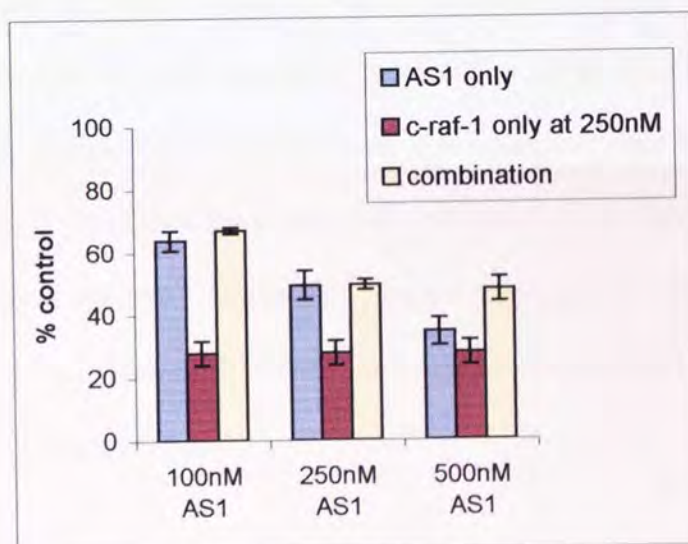

Figure 5.9: Combined effect of C-RAF-1 and AS1 antisense ODNs on A431 cell number over 24 hours.
A431 cells were treated with increasing doses of AS1 ODN and a constant dose of *C-RAF-1* ODN over time complexed with a total concentration of 7.5μM lipofectin. ODN treatment was performed on A431 cells in either 24 or 96 well plates over 24 hours and cell number analysed as in section 2.8.1.2 by crystal violet assay and confirmed by trypan blue exclusion (2.8.1.1). Triplicate experiments were performed and error bars represent n=3 mean ±SD.

Combination effects of AS1 and *C-RAF-1* on A431 cell growth shows that no enhanced effect is observed with the combination treatment at any of the doses applied. Furthermore, the effect of the combined ODNs is less than that found with the C-RAF-1

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alone. It appears from these results that the ODNs, when combined as a treatment, are by some means prevented from exerting their normal singular antisense effects on the cells. Although some inhibition is found, it is of a similar proportion to that found with AS1 alone. If the effect observed was simply similar to that of either of the single doses together then it could be attributed to the fact that the mechanism of inhibition of cell growth is saturated however, it appears here that the ODNS are not able to elicit their effect due to some form of inhibition. To determine whether it was possible that the two oligos would form oligo dimers, an oligo design program was used (primer premier 5, Premier Biosoft). However, ODN dimer formation was not predicted.

Although the combination treatment utilised here was not successful it does not indicate that the use of *C-RAF-1* and AS1 as a combination therapy target is not plausible. It may simply be that in order to gain a combination effect then the issues of delivery of the ODNs separately has to be addressed. For example it may be that the cells require to be treated with one ODN for a period of time prior to the second ODN administration. This would allow the first ODN to enter the cell and exert its effect with the second treatment occurring once the first ODN has been degraded thus allowing it to elicit its effect on any remaining surviving cells.

c) *GRB2* and AS1 Combination

A second ODN was chosen that also targets a gene involved in the *EGFR* signalling pathway, that of *GRB2*. *GRB2* is important for the transformation of fibroblasts by pathways involving *EGFR* and *ERBB2* gene expression and increased expression of these genes has been associated with poor survival in breast cancer (Slamon *et al.*, 1987). Since *GRB2* overexpression has been correlated with increased oncogenic signalling it is believed that it is vital for breast tumorigenesis (Janes *et al.*, 1994).

Tari *et al.*, (1999) have recently used an antisense ODN to target *GRB2* in breast cancer cell lines when delivered using liposomes. The ODN showed a down regulation in proliferation and protein levels of *GRB2* over time compared to that of control cells. The study was concerned with distinguishing the effects of *GRB2* inhibition on breast cancer cells and the effect on both *EGFR* and *ERBB2* signalling. The overall aim of the study in this case was not as a potential treatment but as a method to elucidate the pathways by which *GRB2* plays a role in breast tumorigenesis.

In order to determine the possibility of targeting both *GRB2* and *EGFR* signalling in A431 cells, the effect of *GRB2* antisense molecules used in the study by Tari *et al.*, (1999) was determined. The sequence of the *GRB2* antisense molecule synthesised is documented below.

GRB2 antisense ODN (18mer)

5' ATA TTT GGC GAT GGC TTC 3'

A431 cells were treated with increasing doses of *GRB2*, similar to those used in the previous study (Tari *et al.*, 1999) but were delivered using the system of lipofectin^{TM} optimised here for A431 cells.
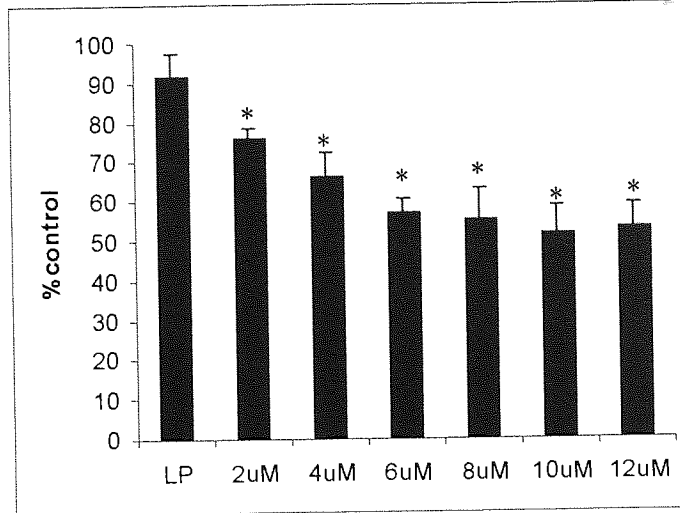
Figure 5.10: Effect of GRB2 antisense ODN on A431 cell number over 24 hours.
A431 cells were treated with increasing doses of *GRB2* antisense ODN over time complexed with 7.5μM lipofectin. ODN treatment was performed on A431 cells in either 24 or 96 well plates over 24 hours and cell number analysed as in section 2.8.1.2 by crystal violet assay and confirmed by trypan blue exclusion (2.8.1.1). Triplicate experiments were performed and error bars represent n=3 mean ±SD. * Represents significant difference (p<0.05) to control cells.

A dose response effect was observed in A431 cells following *GRB2* antisense treatment over 24 hours with lipofectin. Each dose gave a significant effect (p<0.05) on cell number compared to the control samples with the 12μM dose enabling a decrease in cell number of 47% (±6%). Conversely the effect of *GRB2* ODN in breast cancer cells determined by Tari *et al* (1999) was 55% over 5 days thus the effect of *GRB2* knockdown in A431 cells was deemed to be more pronounced. Variations in the results can also be attributed to the variation in cell lines used and delivery methods employed. Again it cannot be determined from this experiment that the effect observed is a true antisense effect without the proper use of controls and protein analysis. However, it is still plausible to determine the efficacy of an antisense combination treatment of *GRB2* and *EGFR* targeting with the use of the antisense ODNs AS1 and *GRB2*.

As with the previous combination treatments the A431 cells were treated with a mix of AS1 and *GRB2* together with the two ODNS complexed with half the maximum

concentration of lipid prior to combination. Cells were treated at AS1 doses of 100nM, 250nM and 500nM with the concentration of *GRB2* remaining constant at 12μM.
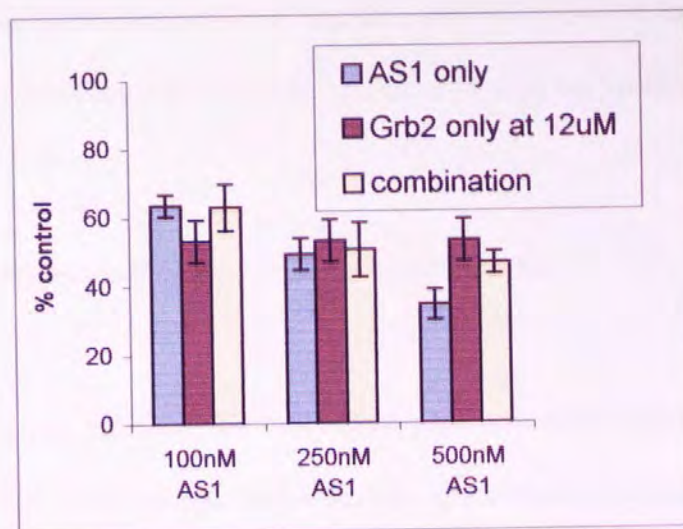
Figure 5.11: Combined effect of GRB2 and AS1 antisense ODNs on A431 cell number over 24 hours.
A431 cells were treated with increasing doses of AS1 ODN and a constant dose of *GRB2* ODN over time complexed with a total concentration of 7.5μM lipofectin. ODN treatments were performed on A431 cells in either 24 or 96 well plates over 24 hours and cell numbers analysed as in section 2.8.1.2 by crystal violet assay and confirmed by trypan blue exclusion (2.8.1.1). Triplicate experiments were performed and error bars represent n=3 mean ±SD.

As previously shown with the combination of *C-RAF-1* and AS1 it appears that no enhanced effect is observed with *GRB2* and AS1 in combination. Further, it is noticed that the effect found with the combination is less than the single effects found by each ODN individually. As postulated with the *C-RAF-1* combination it may be that the antisense mechanism of these ODNs is in some way being inhibited and it is proposed that again oligo dimers are forming where by the ODNs are complementary to each other and are thus binding to each other within the cell. The pairing of the ODNs prevents them from interacting with their target sequence and thus eliciting their effect. Using ODN design programs (primer premier 5, Premier Biosoft) it was predicted that the ODNs AS1 and *GRB2* are capable of forming dimers.

It is concluded that in order for therapies that utilise multiple antisense technologies to be effective the issue of delivery of the ODNs needs to be addressed. It is necessary to deliver the ODNs separately so that they each have time to base pair with their individual sequences and thus elicit a cellular response prior to the ensuing ODN being delivered.

5.2.2 Antisense and Cytotoxic Drug Combinations

For many cancers the main method of treatment used either alone or in combination is that of cytotoxic drugs. These drugs non-specifically inhibit cell growth by various methods including 5-fluorourcil (5FU) inhibition of thymidine formation thereby inhibiting protein synthesis (Parker & Cheng, 1990). Due to the non-specific effects observed with cytotoxic drugs it is important to minimise the dose administered to aid in the reduction of these effects but still allowing a significant biological effect to transpire (Benner et al., 1997).

a) 5FU and AS1 Combination

5-fluorouracil (5FU) is one of the drugs chosen in our study due to its remarkable antitumor activity and the need to reduce the side effects of this agent. Until recently the use of 5FU for treatment of brain tumours was not feasible due to its inability to cross the blood brain barrier. However recently a biodegradable polymer delivery system has been developed that enables sustained delivery of the molecule to the cerebral spinal fluid (CSF) with little systemic toxicity and lower blood levels (Menei et al., 1999). With this potential new advance in brain tumour therapy it was proposed that the combination of a cytotoxic drug such as 5FU and antisense treatment, namely AS1 might aid in providing

an enhanced effect on brain tumour survivability. In order to test this hypothesis the effects of 5FU alone on A431 cell growth first had to be investigated.

A431 cells were incubated with increasing doses of 5FU over a 24-hour period. As 5FU is relatively insoluble in water it was dissolved in the solvent DMSO. In order to ensure that the DMSO has no effect on cell number itself it was used as a control at 0.5%, the maximum amount of DMSO present in any of the sample dilutions used. No delivery agent is required for the administration of 5FU to cells as it is a relatively small lipophilic molecule thus should easily be able to cross the cell membrane.
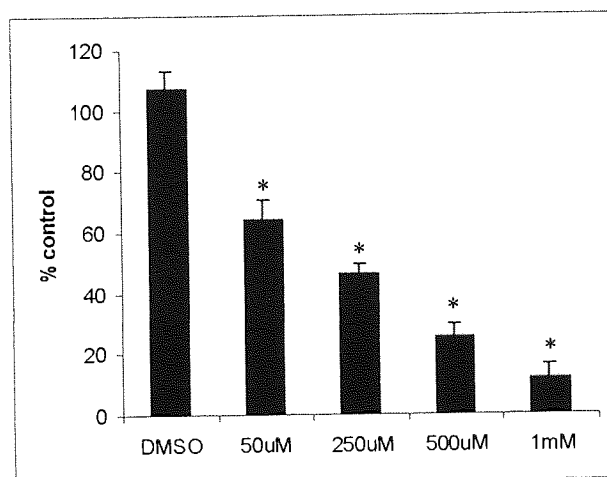
Figure 5.12: Effect of 5FU on A431 cell number over 24 hours.
A431 cells were treated with increasing doses of 5FU over time. Treatment was performed on A431 cells in either 24 or 96 well plates over 24 hours and cell number analysed as in section 2.8.1.2 by crystal violet assay and confirmed by trypan blue exclusion (2.8.1.1). Triplicate experiments were performed and error bars represent n=3 mean ±SD. * Represents significant difference (p<0.05) to control cells.

From the results obtained it can be seen that a dose response curve is present with increasing drug dose correlating with decrease in cell number. A significant (p<0.05) decrease in cell number was observed at all doses present with a 54% (±3%) decrease evident at 250μM. The dose of 5FU required to elicit a decrease in cell number in A431 cells appears to be relatively high compared with levels in HeLa cells found in the low

(<10) micromolar range (Ferguson *et al.*, 1999). This may indicate that A431 cells are naturally chemoresistant to conventional therapies such as 5FU and thus would be an example of the type of scenario where antisense therapy in combination may be advantageous. Combination therapy may allow the resistance of cells to the radiation and chemotherapeutic agents to be decreased thus enabling treatment at much lower, less toxic doses, allowing reduced side effects and increased patient quality of life (Ohnishi *et al.*, 1998).

To evaluate the effect of AS1 in combination with 5FU, the following study was performed. A431 cells were incubated with increasing doses of AS1 for 4 hours in the presence of lipofectin following which the media was removed and fresh media containing 250μM 5FU added.
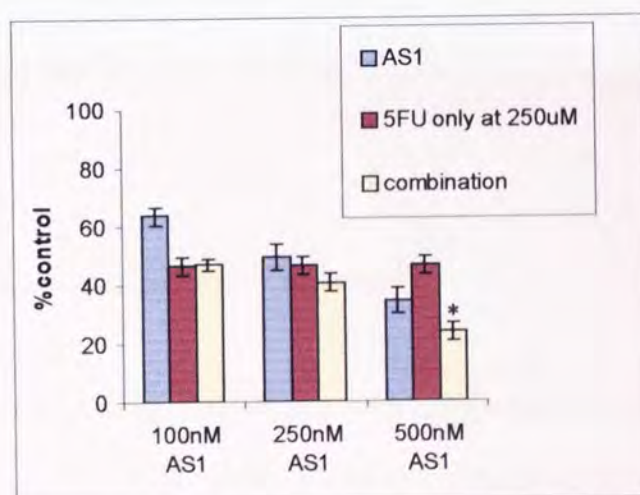
Figure 5.13: Combination effect of 5FU and AS1 on A431 cell number over 24 hours.
A431 cells were treated with increasing doses of AS1 for 4 hours followed by a constant dose of 5FU for a further 24 hours. Treatment was performed on A431 cells in either 24 or 96 well plates over 24 hours and cell number analysed as in section 2.8.1.2 by crystal violet assay and confirmed by trypan blue exclusion (2.8.1.1). Triplicate experiments were performed and error bars represent n=3 mean ±SD. * Represents significant difference (p<0.05) to both treatments alone.

The results obtained show that at a dose of 500nM AS1 the effect on cell growth of both AS1 and 5FU combined is significantly more (p<0.05) than that observed with either treatment alone. Although the combined effect is not a super additive effect, as observed

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when treating colon cancer cells with EGFR antisense ODNs and cisplatin (Ciardiello *et al.*, 2001), a cooperative effect is still observed. When cells were treated with AS1 alone a decrease of 66% was observed at 500nM and with 250mM 5FU a decrease of 54% was found. The combined treatment of these two doses allowed a 77% (±3%) reduction in cell number clearly showing an enhanced cooperative effect of the two drugs. The effect observed is a clear indication that combined treatment of antisense ODNs and cytotoxic drugs are capable of playing a role in future treatment therapies if developed further. A strict treatment regime is required with the precise effective dose for individual cases calculated prior to therapy. With 5FU alone a dose of 500μM was required to give a similar proportion of cell number knockdown thus the use of combination treatment here has allowed the dose of 5FU to be reduced without a reduction in the cytotoxic effect. In a patient this would potentially allow a tumour that is normally relatively resistant to 5FU to be treated as the dose may be below the toxic threshold of the therapy (Leonetti *et al.*, 1999).

b) Temozolomide and AS1 Combination

Due to the promising results found with combination treatment of 5FU a second cytotoxic drug was evaluated for its effect on A431 cell growth both alone and in combination with AS1. Temozolomide is an alkylating agent that when metabolised within the cell can allow methylation of guanine and adenine residues along the DNA sequence. The presence of methyl groups activates the DNA mismatch repair system which upon failure to find a complementary base to the methylated base allows DNA nicks to be present which will prevent the cell from progressing through the cell cycle further (Karran *et al.*, 1993).

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Temozolomide is a drug that shows a broad spectrum of antitumour activity against various types of tumours both *in vivo* and *in vitro* and in preclinical studies showed relatively low toxicity with wide spread tissue distribution including the CNS (for review see Friedman *et al.*, 2000). At present it has entered phase II clinical trials in the treatment of high grade gliomas such as glioblastoma multiforme (Brandes *et al.*, 2000; Brada *et al.*, 2001) and as such is a promising combination therapy to link with *EGFR* antisense targeted therapy.

Initially A431 cells were treated with temozolomide for 24 hours and its effect on cell number observed. The drug was again dissolved in DMSO to aid solubility and thus controls of DMSO only treated cells were used as for the 5FU treatment described earlier.
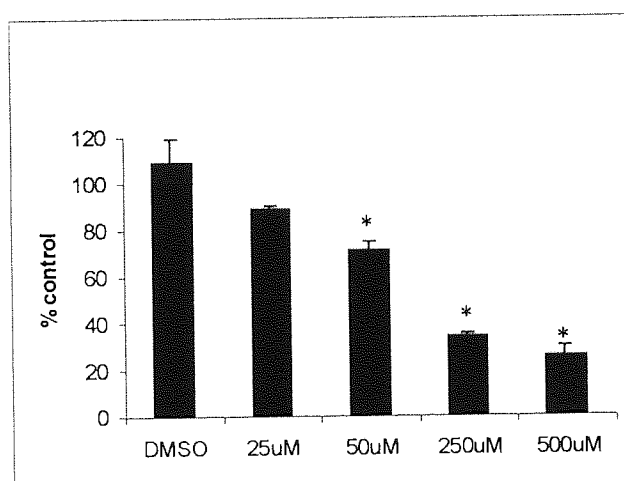
Figure 5.14: Effect of temozolomide on A431 cell number over 24 hours.
A431 cells were treated with increasing doses of temozolomide over time. Treatment was performed on A431 cells in either 24 or 96 well plates over 24 hours and cell number analysed as in section 2.8.1.2 by crystal violet assay and confirmed by trypan blue exclusion (2.8.1.1). Triplicate experiments were performed and error bars represent n=3 mean ±SD. * Represents significant difference (p<0.05) to control cells.

Results show that a significant effect (p<0.05) on cell number compared to that of DMSO only treated controls was observed at all doses greater than 50μM. A431 cells exposed to 250μM temozolomide exhibited a 65% (±0.8%) decrease in cell number compared to control cells. This was a greater effect to that shown with the equivalent dose of 5FU and

similar to that found with 500nM AS1. Doses used were similar to those used in another study on Jurkat cells, a human leukaemia cell line, with effects seen at 250µM over 24 hours of 40% inhibition in cell number compared to control cells (D'Atri *et al.*, 2000). Following the promising results obtained with temozolomide treatment of A431 cells, a combination study of AS1 and temozolomide was undertaken. AS1 dose was varied between 100nM and 500nM whilst the dose of temozolomide remained constant at 250µM. As with the 5FU combination treatment, cells were initially treated for 4 hours with AS1 complexed with lipid followed by a further 24 hours treatment with temozolomide.
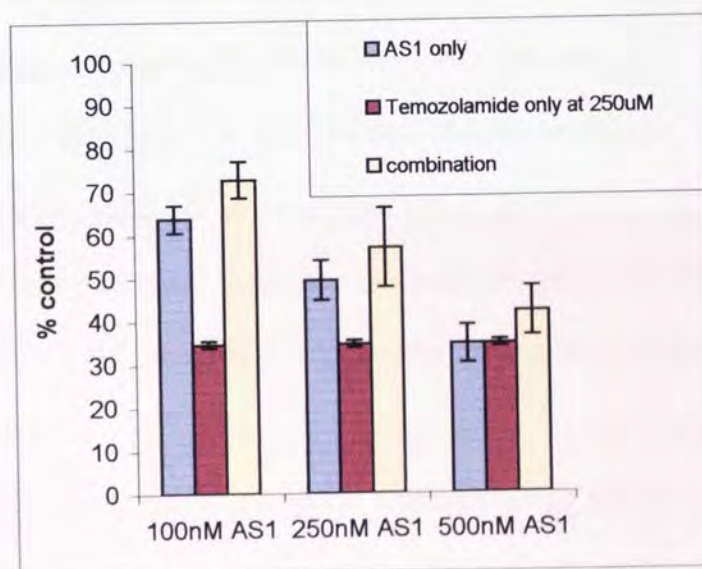
Figure 5.15: Combination effect of temozolomide and AS1 on A431 cell number over 24 hours.
A431 cells were treated with increasing doses of AS1 for 4 hours followed by a constant dose of temozolomide for a further 24 hours. Treatment was performed on A431 cells in either 24 or 96 well plates over 24 hours and cell number analysed as in section 2.8.1.2 by crystal violet assay and confirmed by trypan blue exclusion (2.8.1.1). Triplicate experiments were performed and error bars represent n=3 mean ±SD.

Contrasting to the combined effect found with 5FU and AS1 (figure 5.13) the combined treatment of A431 cells with AS1 and temozolomide did not confer an enhanced effect to

either treatment alone. The effect of AS1 alone at 500nM was 65% and the effect of temozolomide alone at 250μM was 65% but the combined effect of these two doses was only 58% ($\pm$5.7%). This decrease in effect compared to the individual treatments is likely to be as a consequence of the mechanism of action of the temozolomide. As stated previously, temozolomide works by methylating guanosine and adenine residues of DNA, which will cause a structural change in the DNA molecule as well as preventing the base pairing with their respective partners to occur. The mechanism of action of antisense treatment whether it be by an RNase H-type or steric hindrance-type mechanism relies solely on the ODN binding to its specific DNA target by Watson-Crick base pairing. As such any change in either the ODN sequence or the target DNA sequence will render the antisense therapy useless, as it will not be able to bind. It is plausible therefore that in this case the temozolomide is performing its function by methylating bases but unfortunately this is stopping the antisense molecule from binding. Temozolomide could methylate the target sequence as well as the antisense ODN, thus preventing the interaction of the two molecules and subsequent inhibition of EGFR synthesis.

Future therapy that relies on the combination of antisense ODNs and drugs that inhibit cell growth by means of modifying DNA needs to be carefully considered. It may be possible with the use of suitably designed treatment regimes to elicit a combined effect with such therapies (D'Atri *et al.*, 2000). It may be that with a heterogeneous tumour where some of the tumour is highly resistant to alkylating agents that the remaining resistant cells following initial drug treatment can be targeted with antisense ODNs, but this would be dependant on molecular analysis of the tumour present.

5.3 Conclusions

In this chapter the potential therapy of antisense molecules designed by chip technology in chapter three was evaluated. Although these ODNs have shown good prospects for the targeting and down-regulation of their *EGFR* target in A431 cells, their efficacy in gliomas had not been addressed.

Two primary tumour cell lines, IN859 and IN2045, were derived from biopsy samples taken from patients exhibiting brain tumours and used to test the efficacy of AS1 and AS2 on cellular proliferation. Results showed that both AS1 and AS2 were capable of reducing cell number within these lines compared to control samples but that the scope of inhibition found varied greatly between the cell lines. Protein analysis of the EGFR levels was inconclusive with the levels of expression too low to be detected. It is postulated that the differences between the cell lines was dependent on the amount of EGFR expressed and that the IN2045 cell line, which exhibited less response to treatment, has lower EGFR levels than the IN859 cell line. The vast heterogeneity of a glioma tumour has been shown (Shapiro & Shapiro, 1998) and thus it may be that in the IN859 cell line developed the original tumour had more cells over-expressing EGFR than in the IN2045 tumour.

Due to the heterogeneity of gliomas it is unlikely that one treatment regime alone will be sufficient to allow inhibition/ death of all of the tumour cells. Thus it is plausible that successful treatment of brain tumours will require a combination of therapies. These could be complete antisense treatment regimes where by different ODNs are used to

target problem sequences within the cell or as a combination of more conventional therapies, such as chemotherapy and antisense treatment.

Combination effects of both AS1 and AS2 on A431 cell growth showed an enhanced effect compared to single treatments alone. This would enable the overall dose of antisense ODNs to be reduced with little or no reduction in efficacy. It may be that the uptake of ODN into the cell population is wider spread with both ODNs compared to a single ODN alone or it may be that upon binding of one ODN the second target site becomes even more accessible to the subsequent ODN.

Combination effects using AS1 and either *C-RAF-1* or *GRB2* showed less effect than the single ODN treatments alone. *C-RAF-1* ODN developed by Monia *et al.*, (1996) and now in clinical trials, showed a decrease in cell number over 24 hours alone but no effect when combined with AS1. Similarly *GRB2* showed an inhibition of cell growth alone over 24 hours but no effect in combination. It is postulated that with both of these ODNs the two ODN sequences are capable of reverse complementarity thus preventing each other from binding to the target site. To overcome this problem it is necessary to design a treatment regime where by one ODN can exert its effect prior to the second ODN entering the cell thus preventing the ODNs from interacting with each other. It may be possible to use a delivery system such as biodegradable microspheres whereby the first ODN is released quickly to the target cells but the second ODN is released at a slower rate allowing the first ODN to exert its effect (Einmahl *et al.*, 1999).

Finally the combination of AS1 with the cytotoxic drugs, 5FU and temozolomide, was investigated. These drugs have shown great potential for the treatment of brain tumours but as with most chemotherapeutic agents exert toxic side effects. Certain tumours can also develop resistance to cytotoxic drugs and thus the areas of the tumour that are resistant need to be targeted using an alternative therapy. The effect of both 5FU and

temozolomide on A431 cell growth was by a dose dependent effect where an increase in drug concentration caused a decrease in cell number. Combination of AS1 with 5FU allowed an increased effect to be observed than with either treatment alone indicating that the two treatments were cooperatively acting on the cell. The outcome of this increased effect meant that the cells could be treated with a much lower dose of 5FU than normally required to observe the same extent of cell inhibition. This would mean that the side effects of the drug would be decreased as the dose of treatment required would be much lower. Unfortunately, similar effects were not observed with the combination treatment of temozolomide and AS1, which exerted little effect on cell number compared to that of either drug alone. It is proposed that the method by which temozolomide inhibits cell growth is also acting to inhibit antisense effect by AS1.

Overall the studies performed here have shown that some combination therapies can provide advantageous effects on growth inhibition of tumorigenic cells but that others require much more development before they can be classed as effective treatments. It is likely that the use of antisense combinations will be necessary in treating such tumours as gliomas due to the high heterogeneity of the tumours and the sensitivity of surrounding cells to conventional therapies. Delivery mechanisms and designated treatment regimes are likely to play a major role in any future therapy for glioma combination treatment.

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Appendix

Presentations

Petch, A., Sohail, M., Southern, E.M., Akhtar, S. (Jun 1999). DNA Chip technology as a novel technique for designing effective antisense oligodeoxynucleotides against *EGFR* mRNA. (CRS, Boston, USA.)

Petch, A. Sohail, M. Southern, E.M. Akhtar, S. (Sep 1999). Combination therapy with conventional anti-cancer drugs and antisense Oligodeoxynucleotides against *EGFR* mRNA. (BPC, Cardiff, UK.)

Petch, A. Sohail, M. Southern, E.M., Darling, J., Akhtar, S. (Sep 2000). Effect of antisense ODNs on cell proliferation of human primary brain tumour cell lines. (BPC, Birmingham, UK.)