Use of ribozymes and antisense oligodeoxynucleotides to investigate mechanisms of drug resistance

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Abstract

Chemotherapy can cure a number of human cancers but resistance (either intrinsic or acquired) remains a significant problem in many patients and in many types of solid tumour. Combination chemotherapy (using drugs with different cellular targets/mechanisms) was introduced in order to kill cells which had developed resistance to a specific drug, and to allow delivery of a greater total dose of anti-cancer chemicals by combining drugs with different side-effects (Pratt *et al.*, 1994). Nearly all anti-cancer drugs kill tumour cells by activating an endogenous bio-chemical pathway for cell suicide, known as programmed cell death or apoptosis.

Introduction

Cancer cells can generate variants which are multi-drug resistant (MDR) exhibiting diminished sensitivity to a broad range of chemically and mechanistically unrelated drugs (Clynes, 1994; Ravidin, 1995). Most research in this area to date has focused on broad-spectrum drug-efflux pumps such as p-170 (Croom, 1993) and MRP (Ishikawa *et al.*, 1996; Canitrot *et al.*, 1996) and on alterations in levels of drug-metabolising enzymes such as glutathione-S-transferases (Moscow and Dixon, 1993) or drug targets such as topoisomerase II (Nitiss and Beck, 1996). While these proteins undoubtedly play a role in MDR, and have been targets for clinical trials of resistance modulators (Ferry *et al.*, 1996) they are almost certainly not the only determinants of MDR *in vivo*. The range of drugs affected by these efflux pumps (including anthracyclines, vinca alkaloids and epipodophyllotoxins) is narrower than the broad-range MDR often seen in non-responsive cancer patients. While searching for *in vivo* modulators of MDR the observation that malignant cells commonly become resistant to chemotherapeutic drugs while normal cells rarely do suggested that

molecular processes involved in malignancy, such as oncogene activation, may play a role in drug resistance (Sklar and Prochownik, 1991). Adding to this observation is the fact that many oncogenes have also been found to influence the cell's capacity to undergo apoptosis, the process of active cell death induced by many chemotherapeutic drugs.

A number of oncogenes encode mutant forms of cell surface growth factor receptors which have been shown to influence apoptosis and chemotherapeutic drug responses in cells and tissues. Many cell lines over-expressing c-erbB-2 (a gene which encodes a 185 kDa transmembrane glycoprotein that is amplified and over-expressed in about a quarter of breast carcinomas) exhibit an increased resistance to chemotherapeutic drugs (Sabbatini *et al.*, 1994; Yu *et al.*, 1996; Zhang and Hung, 1996). Signal transducers such as the Ras and Raf proteins which play a fundamental role in cellular proliferation affect resistance levels in *in vitro* systems (Isonishi *et al.*, 1991; Sabbatini *et al.*, 1994); as does over-expression of nuclear oncogenes, such as c-fos, which act as transcription factors (Scanlon *et al.*, 1991; Funato *et al.*, 1992; Zhao *et al.*, 1995).

Many members of the bcl-2 family function similarly to bcl-2 (i.e. they inhibit apoptosis); these include bcl-xL, mcl-1, A1, Bfl-1, cl-w, Bbag-1. Several members of the family do the opposite (i.e. promote apoptosis) including bax, bcl-xs, bad, bak, bik, Hrk and Bid. The ratio of expression of pro-apoptotic and anti-apoptotic members of the bcl-2 family determines how easily a cell will undergo apoptosis when confronted with an apoptotic stimuli e.g. chemotherapeutic drug. Many malignant cells develop defects in the regulation of these genes that control apoptosis rendering them resistant to the induction of apoptosis by a wide variety of stimuli including chemotherapeutic drugs. A variety of experiments have provided evidence that elevations in Bcl-2 expression cause resistance to chemotherapeutic drugs while decreases in bcl-2 expression promote apoptotic responses to anticancer drugs (Boulakia *et al.*, 1996; Datta *et al.*, 1996; Ibrado *et al.*, 1996a; Ibrado *et al.*, 1996b). Over-expression of the anti-apoptotic members of the bcl-2 family has been shown to create a cellular environment that is permissive for the accumulation of mutants (Cherbonnel-Lasserre *et al.*, 1996; Minn *et al.*, 1996). Thus damaged tumour cells that over-express these genes can acquire secondary mutations that can lead to the emergence of other mechanisms of drug resistance (e.g. over-expression of *mdr1*) and tumour progression via oncogene activation or tumour suppressor gene activation.

The role of the Bcl-2 family in drug resistance is well documented (Kitada *et al.*, 1994; Teixeira *et al.*, 1995; Wagener *et al.*, 1996) as are other apoptosis affecting genes such as p53 (reviewed in Kellen, 1994) and c-myc (Kashani-Sabet *et al.*, 1990; Sklar and Prochownik, 1991; Mizutani *et al.*, 1994). The anti-apoptotic oncogene Bcr-abl has been definitively shown to block cell death caused by anticancer drugs in neutrophils (McGahon *et al.*, 1994). The tyrosine protein kinase c-Abl is a JNK (c-Jun N-terminal Kinase) activator, which when activated phosphorylates the c-Jun transcription factor (which forms the AP-1 complex) and other transcription factors including p53, thus indicating a potential role for the JNK pathway in multidrug resistance. CD95 (Fas/APO-1) ligand signalling (which induces a cell death pathway involving caspase activation) may also activate JNK (Yang *et al.*, 1997).

A variety of approaches has been attempted to overcome drug resistance. An increasing number of compounds capable of reversing MDR continues to be identified (Beck, 1991; Ford and Hait, 1990; Bel-

lamy, 1996; Bosch and Croop, 1996; Bates *et al.*, 1996). Several types of strategy have been utilised to modulate the MDR phenotype both *in vitro* and *in vivo*. Inhibition of p-glycoprotein has been achieved by using specific drugs such as the calcium channel blocker verapamil and the immunosuppressive agent cyclosporin A (Slater *et al.*, 1986; Sonneveld *et al.*, 1992; Tsuruo *et al.*, 1981; Dalton *et al.*, 1989; Salmon *et al.*, 1991; Futscher *et al.*, 1992). New quinoline derivatives MS-209 and MS-073 have also shown their ability to modulate the MDR phenotype; studies are currently in progress to further investigate their *in vivo* use (Sato *et al.*, 1991; Sato *et al.*, 1995). Difloxacin has been shown to reverse *in vitro* the MDR phenotype (Gollapudi *et al.*, 1995). However, toxic effects of these compounds has limited their clinical use. Modulation of intracellular glutathione or GST (using buthionine sulfoximine (Bellamy *et al.*, 1991; Griffith, 1982; Ozols *et al.*, 1987; O'Dwyer *et al.*, 1991, 1992)) has also been attempted. However, unacceptable side-effects of a number of these compounds have been the limiting factor in administration of the reversal agent in a number of protocols. Novel approaches to circumventing MRP-mediated drug-resistance have been recently reported (Duffy *et al.*, 1998). Recently a number of molecular approaches to reverse MDR have been developed; antisense and ribozyme constructs have been utilised to reverse drug resistance by decreasing *pgp* (Efferth and Volm, 1993; Thierry *et al.*, 1993; Quattrone *et al.*, 1994; Hanchett *et al.*, 1994; Kobayashi *et al.*, 1993, 1994; Holm *et al.*, 1994; Scanlon *et al.*, 1994), MRP (Canitrot *et al.*, 1996), c-fos (Scanlon *et al.*, 1991; Ohta *et al.*, 1996; Scanlon *et al.*, 1994) and bcl-2 (Kitada *et al.*, 1994). Another strategy for overcoming resistance is to specifically target over-expressing cells with anti-MDR monoclonal antibodies (Tong *et al.*, 1989). As most chemosensitizing agents affect only one component of these multi-factorial MDR mechanisms their effectiveness is limited (Dalton *et al.*, 1989; Miller *et al.*, 1991). The development of combination therapy protocols involving several different strategies (e.g. chemosensitizers, antibodies, oligonucleotides or ribozymes) may improve overall efficacy.

Use of Ribozymes to Study Drug Resistance

Ribozyme technology

Ribozymes are RNA molecules with site-specific ri-

bonuclease activity (Cech, 1990; Altman, 1987; Symons, 1992). Naturally occurring ribozymes are classified into 6 groups:

- 1) Ribozymes derived from self-splicing of Tetrahymena group I introns (Cech *et al.*, 1981; Kruger *et al.*, 1982);
- 2) RNA components of RNase P (Gurrier-Tahada *et al.*, 1983);
- 3) hammer-head ribozymes (Uhlenbeck, 1987);
- 4) hairpin ribozymes (Buzayan *et al.*, 1986);
- 5) genomic and anti-genomic Rnase of hepatitis δ virus (Perotta and Been, 1992);
- 6) RNA transcripts of mitochondrial DNA plasmid of *Neurospora* (Symons, 1994).

Because of its simple self-cleavage domain and small size the hammer-head ribozyme is frequently used in cell systems.

Ribozymes contain stretches of nucleotides that base-pair with complementary RNA regions and have a catalytic section analogous to the active site of a protein enzyme that cleaves the bound RNA while the base-pairs hold it in place (Uhlenbeck, 1987; Haseloff and Gerlach, 1988; Cameron and Jennings, 1989). The hammer-head ribozyme can cleave the 3' end of the triplet NUX where N is any nucleotide and X is C, A or U.

Theoretically ribozymes can be engineered to cleave any RNA species site-specifically in the background of cellular RNA. This cleavage renders the mRNA unstable and abrogates protein expression. mRNA coding for any protein associated with a disease state can be cleaved selectively by ribozymes expressed from gene therapy vectors and as such ribozymes might be useful as antigene therapeutic agents. *mdr1* ribozymes have potential not only as therapeutic tools but also as a laboratory tool for the elucidation of the relationship between mRNA expression and phenotypic characteristics.

Ribozymes have distinct advantages over proteins when considering their use in therapeutic applications:

- 1) RNA is less likely to evoke an immune response than expression of a foreign or altered protein;
- 2) ribozymes are small facilitating their insertion into gene therapy vectors;
- 3) multiple ribozymes targeting several mRNAs or multiple regions of a virus can be inserted into vectors.

A number of technical hurdles still need to be overcome before ribozymes can be used as mediators of gene therapy:

- 1) transcription units must be designed that will accumulate sufficient numbers of ribozymes;
- 2) ribozymes must be chosen that will anneal to an accessible region of the target mRNA;
- 3) the expressed ribozyme must maintain sufficient catalytic activity to cleave the target mRNA before it is translated;
- 4) appropriate vectors for the particular diseases/organ must be chosen.

Appropriately engineered ribozymes must demonstrate specific inhibition of gene expression in appropriate preclinical disease models before human *in vivo* applications can be contemplated.

Many studies have been carried out to optimise ribozyme structure for use as therapeutic agents for gene therapy. DNA has been substituted for RNA in the various nucleotides of the ribozyme in order to construct more efficacious and stable ribozymes (Taylor *et al.*, 1992; Shimayana *et al.*, 1993). Sequences of substrate RNA and their secondary structure are important for catalytic efficiency (Fedor *et al.*, 1990). Adding bases to the ribozyme flanking sequences increased site specificity but decreased the dissociation time of the ribozyme substrate (Herschlag *et al.*, 1991); a 12 base optimal length for the flanking sequences of the ribozyme has been discovered (Bertrand *et al.*, 1994). A number of targets for ribozyme mediated gene modulation have been identified, ranging from cancer cells to infectious diseases.

Ribozyme technology in the study of MDR

Several groups have demonstrated ribozyme-mediated modulation of MDR in human cancer cells. Scanlon and co-workers report the reversal of the MDR phenotype in human ovarian carcinoma cells using either an *mdr1* ribozyme or a *fos* ribozyme (Scanlon *et al.*, 1994). Using an anti-*mdr1* ribozyme designed to cleave the CUC sequence of codon 880 (a target site between 2 ATP binding sites which may play a role in the pgp pump) resistance in a human ovarian carcinoma cell line (16.6 fold resistant to actinomycin D and over-expressing *mdr1*) was completely reversed to the sensitive level. *mdr1* expression was reduced and actinomycin D intracellular transport level was increased.

The anti-*fos* ribozyme (which targets the GUC sequence of codon 309) reversed actinomycin D resistance more quickly than did the *mdr1* ribozyme. This may suggest that *c-fos* may modulate the expression of genes other than *mdr1* which also contribute to the MDR phenotype. In this cell line the anti-*fos* ribozyme down-regulated not only *c-fos* but also the expression of *mdr1* and topoisomerase I (the *mdr1* promoter has an AP-1 binding site (Teeter *et al.*, 1991)).

The *mdr1* ribozyme has been shown to be effective in a number of other MDR cell types. Holm and co-workers report the reversal of daunorubicin resistance in resistant (1600 fold) human pancreatic carcinoma cells (Holm *et al.*, 1994). The ribozyme-containing cells were 300 fold more sensitive to daunorubicin than the parent resistant cells.

In two lung cell lines the ribozyme has a similar effect; *mdr1* ribozyme transfectants of two MDR lung cell lines (DLKP-A and SKMES-1ADR) were found to be more sensitive to adriamycin, vincristine and VP-16 (Daly *et al.*, 1996). The anti-*mdr1* ribozyme does not result in complete reversal of resistance to the level of the sensitive parent. Possibly the level of *mdr1* mRNA remaining in the *mdr1* ribozyme transfectants is capable of mediating drug resistance and/or other mechanism of resistance may be present in these lines.

Kobayashi and colleagues have designed ribozymes directed at other areas of the *mdr1* mRNA transcript (Kobayashi *et al.*, 1994). They designed two hammer-head ribozymes; one targeted at codon 179 and the other at codon 196. In *in vitro* studies the ribozyme targeted at the 196 codon proved most effective. This ribozyme was then used in transfections to target *mdr1* mRNA in an acute leukemia cell line. The ribozyme down-regulated *mdr1* expression as well as pgp expression. Vincristine resistance was decreased from 700 to 20 fold. Once again complete reversal of drug resistance did not occur. This leukemic cell line was highly drug resistant and as such possibly not enough ribozyme was expressed to down-regulate the over-expressed *mdr1* mRNA or the resistance mechanism in these cells could be multi-factorial.

Bertram and co-workers have studied the ribozyme-mediated reversal of drug resistance in a human colorectal carcinoma (LoVo/Dx) cell line resistant to doxorubicin (LoVo/Dx^R) (using a ribozyme directed against *mdr1* mRNA) (Bertram *et al.*, 1995). These authors designed two ribozymes against *mdr1*, ribozyme 1 (targeting the CUC sequence at position 2429) and ribozyme 2 (targeting the GUC sequence at position 2440). *In vitro* these ribozymes reduced the *mdr1*

mRNA from the LoVo/Dx^R cells by up to 80%. Modified ribozymes (containing fluoro and allyl substituted bases to increase stability against ribonucleolytic attack) reduced chemo-resistance of these cells by up to 50%.

Using three *mdr1* ribozymes (ribozyme 1 targeting position 2429, ribozyme 2 targeting position 2440 and ribozyme 3 targeting 2408) in *in vitro* assays Palfner and co-workers (1995) assayed conditions such as ribozyme-to-target ratio, pH, MgCl₂ concentration and incubation time on cleavage efficiency (Palfner *et al.*, 1995). Their most efficient ribozyme cleaved 91% of an *in vitro* transcribed *mdr1* mRNA transcript.

Ribozyme studies can also be used in functional assays of multi-drug resistance. Eijdem and co-workers report the down-regulation of *mdr1* mRNA using an *mdr1* ribozyme targeted at codon 196 (Kobayashi *et al.*, 1994; Eijdem *et al.*, 1995) in a human non-small cell lung cancer cell line SW-1573 selected in a low concentration of doxorubicin. In a clone having reduced *mdr1* mRNA level there was no detectable change in sensitivity to drug which suggests that *mdr1* does not contribute to drug-resistance in these cells. These authors have concluded that resistance in this cell line is associated with the presence of an altered form of MRP.

Increased expression of *c-fos* is often found associated with drug-resistance and several of these drugs (e.g. cisplatin) are not substrates for *mdr1* or MRP (Scanlon *et al.*, 1994; Hollander and Fornace, 1989). *fos* is believed to mediate its effects through transcriptional activation, after interaction with the Jun protein, to form the AP-1 complex. This complex affects proliferation, apoptosis and drug resistance through transcriptional activation of genes via AP-1 elements in their regulatory regions. The A2780 ovarian carcinoma cell line resistant to cisplatin has been shown to exhibit *c-fos* overexpression as well as the over-expression of *c-myc*, *H-ras*, thymidylate synthesis, DNA polymerase B and Topoisomerase I (Scanlon *et al.*, 1990, 1991; Kashani-Sabet *et al.*, 1990). This may suggest that *c-fos* is involved in cisplatin resistance by directing expression of enzymes carrying out DNA synthesis and repair processes. Tumour tissue from a patient with colon carcinoma failing cisplatin/5-fluorouracil treatment revealed a similar pattern of gene expression to the resistant A2780 subclone (Kashani-Sabet *et al.*, 1990a). These data suggest that the *c-fos* gene regulates downstream enzymes associated with DNA synthesis and repair and may play a central role in cisplatin resistance.

It has already been discussed how anti-*fos* ribozymes are effective in lowering resistance in classic MDR cell lines. *fos*-ribozymes have also been shown to be effective in altering resistance in non-classic MDR, e.g. resistance to cisplatin. Cisplatin is one of the most widely used anti-cancer agents and its multifactorial mechanisms of resistance pose serious clinical problems in cancer chemotherapy (Ishida *et al.*, 1995). Studies in cisplatin-resistant cell lines support the importance of the *c-fos* oncogene in maintaining the drug-resistant phenotype (Scanlon *et al.*, 1989). A hammer-head ribozyme against the *c-fos* gene has been investigated in a cisplatin resistant line (Scanlon *et al.*, 1991; Funato *et al.*, 1992). The cisplatin resistant human ovarian carcinoma A2780 subclone (A2780DDP, 10-fold resistant to cisplatin) was transfected with an anti-*fos* ribozyme and was rendered sensitive to the antineoplastic effects of cisplatin (as well as camptothecin, 5-fluorouracil and azidothymidine to which A2780DDP cells are cross-resistant). The ribozyme transfectant was found to have down-regulation of *c-fos* gene expression as well as down-regulation of *c-fos* responsive genes such as DNA polymerase B, Topoisomerase I and metallothionein IIA. Down-regulation of *cfos* may reverse drug-resistance by several mechanisms involving DNA synthesis as well as pgp.

Funato and co-workers (1997) prepared a hammer-head ribozyme to selectively cleave *fos* mRNA. The ribozyme was transfected into implanted human colon cancer cells SW480DDP and SW620DDP (which over-express the *fos* gene and are resistant to cisplatin treatment) and it reduced the expression of the *fos* gene *in vivo* and also increased cell sensitivity to cisplatin.

Ribozyme technology in the study of apoptosis

There is increasing interest in understanding the mechanisms by which cancer cells regulate their susceptibility to induction of apoptosis (programmed cell death) on exposure to chemotherapeutic drugs and other therapeutic agents. An understanding of the molecular mechanisms involved in apoptosis could be used to sensitise tumours to therapy. Ribozymes targeted against genes involved in apoptosis could play a role in such a therapeutic strategy.

Ribozyme-mediated reduction of the *c-Ha-ras* oncogene expression in a cultured human bladder carcinoma cell line (EJ) resulted in a reduction in tumour formation and invasion when these cells were im-

planted in nude mice (Kashani-Sabet *et al.*, 1992), thus supporting the role of *c-Ha-ras* in tumourigenesis. Feng *et al.* (1995) have also made use of a hammer-head ribozyme designed to cleave the mutant sequence in codon 12 of the activated *H-ras* oncogene transcript. By using a recombinant adenovirus as a gene cassette for the *H-ras* ribozyme, high efficiency reversion of the neoplastic phenotype in mutant *H-ras* expressing tumour cells was achieved. Eastham and Ahlering (1996) undertook a study to evaluate the ability of a *H-ras* ribozyme to alter the malignant phenotype of an invasive human bladder cancer cell line, EJ. Cell lines with and without the anti-*ras* ribozyme were examined for their malignant potential in nude mice and the results suggest that the invasive phenotype was delayed, but not abolished, when the anti-*ras* ribozyme was present.

The unique *Bcr-abl* fusion gene, which is believed to play a crucial role in the pathogenesis of CML, has offered scientists an opportunity to specifically target cancer cells, while sparing their normal neighbours, with ribozyme technology. In the CML cell line K562, Lange and co-workers (1994) were able to inhibit synthesis of the p210 *Bcr-abl* protein by a ribozyme which was about twofold more effective than the corresponding antisense molecule. Leopold *et al.* (1995) used a multi-unit ribozyme to target the *bcr-abl* gene and found that the level of *bcr-abl* mRNA was reduced 3 logs when transfected via folate receptor-mediated uptake into transformed 32D cells.

CD95(Fas/APO-1)-ligand has also been a target for ribozyme technology (Du *et al.*, 1996). An anti-p53 ribozyme has been utilised by Cai and co-workers (1995) to cleave the p53 pre-messenger RNA and has been shown to efficiently reduce the level of endogenous mutant p53 mRNA. Retrovirus-mediated transduction of a hammerhead ribozyme designed to cleave unspliced p53 at codon 187 near the boundary of intron 5 and intron 6 reduced the level of mutant p53 RNA and protein in the human H226Br lung cancer cell line, which contains a homozygous p53 mutation at codon 254. Expression of the ribozyme significantly suppressed the growth of the H226Br cells in culture.

Dolnikov *et al.* (1996) designed two hammer-head ribozymes that specifically cleaved the *v-myc* (but not the *c-myc*) transcript *in vitro* in the myelomonocytic cell line, tEMmyc4, which has enforced *v-myc* over-expression (via transfection of the cDNA). The subsequent reduction of *v-myc* expression in ribozyme-expressing cells was associated with abrogation of hormone-induced apoptosis.

Dorai *et al.* (1997a, b) have recently reported the use of a hammerhead ribozyme against bcl-2. The ribozymes were introduced into cultured prostate cancer cells, LNCaP. Results found demonstrated that the functional ribozyme was able to degrade bcl-2 mRNA and protein levels within 18 h of treatment. The activity was sufficient to induce apoptosis in a low bcl-2 expressing variant of LNCaP, but not in the high bcl-2 expressing variant (Dorai *et al.*, 1997b). The results obtained in the other study (Dorai *et al.*, 1997a) strongly support the ability of a potential anti-bcl-2 ribozyme therapy to synergise with other agents in inducing apoptosis of hormone-resistant human prostate cancer cells.

Use of Antisense Oligodeoxynucleotides to Study Drug Resistance

Antisense technology

The notion of using specific oligonucleotides for the modulation of gene expression surfaced two decades ago when Zamenick and Stephenson inhibited the replication of the Rous Sarcoma virus (Zamenick and Stephenson, 1978). They added, to the medium of chick fibroblasts in tissue culture, a synthetic piece of DNA to block the circularisation step by hybridising specifically with the 3' end of the viral RNA in a competitive way. It inhibited the formation of new virus, and also prevented the transformation of chick fibroblasts into sarcoma cells.

The term 'antisense' ascribed to a nucleic acid sequence, refers to the fact that it forms complementary base pairs to a part or all of a genetic coding sequence which is termed the 'sense' strand. Thus once all or part of the sequence a target gene is known, an antisense molecule can be designed which specifically binds to this gene alone. In addition to this hydrogen bonding, the affinity of the antisense molecules to their targets are affected by base-stacking in the double helix of the oligonucleotides and also the ionic strength of the system (Crooke and Bennett, 1996). Affinity between the antisense oligonucleotide and target polynucleotide increases as the length of the antisense molecule increases, due to the increased hydrogen bonding between bases and stacked pairs (Crooke and Bennett, 1996). Theoretically, at least, this allows for the design of drugs to attack any unwanted or mutated form of a gene, and leave the normal copy of the gene untouched, even if the two forms differ by only a sin-

gle base pair or oligonucleotide (Crooke and Bennett, 1996; Milligan *et al.*, 1994; Giles *et al.*, 1995).

Antisense molecules can consist of relatively short synthetic oligonucleotides introduced into cellular systems by various means (Stein *et al.*, 1993; Brysch and Schilingensiepen, 1994; Helene, 1994; Scanlon *et al.*, 1995; Zon, 1995; Crooke and Bennett, 1996; Wagner and Flanagan, 1997). This form is currently the most widely used and entails the use of DNA and RNA based oligonucleotides or combinations of both. Or, alternatively, they can consist of a whole gene, or a specific fragment of a gene, cloned into an expression vector in a reverse orientation and transfected into a cell, where it is expressed as antisense RNA either endogenously or upon stimulation (Scanlon *et al.*, 1995; Branch, 1996; Tolume *et al.*, 1996; Zhang, 1996; Sczakiel, 1997).

The mechanisms by which interactions of antisense oligonucleotides with nucleic acids may induce biological effects are quite complex. The most basic mode of action of antisense is an occupancy-only one (Crooke and Bennett, 1996). The antisense acts as a classic competitive antagonist by binding to specific sequences, inhibiting the interaction of the RNA or DNA with proteins, other nucleic acids or factors required for the essential steps in the intermediary metabolism of the RNA or its utilisation by the cell. Another mechanism is the inhibition of excision of introns or 'splicing', which is a key step in the intermediary metabolism of most mRNA molecules (Crooke and Bennett, 1996; Sharma and Narayanan, 1995; Neckers *et al.*, 1992). The mechanism for which the majority of oligonucleotides have been designed to date is to cause translational arrest by binding to the translation initiation codon or alternatively to bind to areas in the coding region to attempt steric hindrance of ribosome progression along the mRNA (Brysch and Schilingensiepen, 1994; Crooke and Bennett, 1996; Ohkawa *et al.*, 1996; Bouffard *et al.*, 1996; Probst and Skutella, 1996). Although the ribosomal machinery is quite powerful and tends to sweep away most obstacles in its path, targeting the AUG initiation codon where the ribosomes first begin translational has been shown to be a very effective target (Vasanthakumar and Ahmed, 1989; Jaroszewski *et al.*, 1990; Rivoltini *et al.*, 1990; Clynes *et al.*, 1992; Corrias *et al.*, 1992; Efferth *et al.*, 1993; Thierry *et al.*, 1993; Quattrone *et al.*, 1994b; Bertram *et al.*, 1995; Nakashima *et al.*, 1995; Alahari *et al.*, 1996; Cucco and Calabretta, 1996; Liu *et al.*, 1996; Sola and Colombani, 1996;

Stewart *et al.*, 1996; Hirtake *et al.*, 1997; Li *et al.*, 1997).

One of the most important mechanisms of action of DNA based-antisense targeted to RNA is the activation of ribonuclease H (RNase H) (Brysch and Schlingensiepen, 1994; Crooke and Bennett, 1996; Helene, 1994; Bouffard *et al.*, 1996; Ohkawa *et al.*, 1996; Sharma and Nayaran, 1995; Wagner and Flanagan, 1997; Giles *et al.*, 1995; Branch, 1996). RNase H is an ubiquitous enzyme that selectively cleaves the RNA component of RNA-DNA duplexes. Other mechanisms of inhibition of translation include interference with secondary structures, such as stem loops, (Vickers *et al.*, 1991; Ecker *et al.*, 1992; Thierry *et al.*, 1993; Crooke and Bennett, 1996; Liu *et al.*, 1996; Tolume *et al.*, 1996), inhibition of 5' capping (Baker, 1993; Alahari *et al.*, 1996; Stewart *et al.*, 1996; Crooke and Bennett, 1996) and interference with 3' polyadenylation (Chiang *et al.*, 1991; Alahari *et al.*, 1996; Stewart *et al.*, 1996). Oligonucleotides conjugated to alkylating and photoactivable alkylating species have been synthesised. These can then inhibit the target DNA by covalently modifying them, rendering them non-functional (Webb and Mateucci, 1986; Crooke and Bennett, 1996). Activation of mRNA breakdown is not universal, however; Probst and Skatella (1996) found elevation of specific mRNAs by antisense, but not by sense treatments.

Numerous chemical modifications have been made to the oligonucleotide backbones and sugar bases to render them more nuclease resistant and give them greater affinity to their targets. The earliest modifications involved substituting the non-bridging oxygen atoms in the internucleotide bonds with either a methyl or a sulphur group to give methylphosphonate and phosphorothioate oligodeoxynucleotides respectively (Jayarman *et al.*, 1981; Marus-Sekura *et al.*, 1987; Matsukura *et al.*, 1987). This made the oligonucleotides more resistant to nuclease degradation, which was a problem for natural phosphodiester oligonucleotides (Wickstrom, 1986), thus extending the half-life of the oligonucleotides and improving their efficacy. In the methylphosphonate substitution, the negative charge of the oligonucleotide is eliminated, and exhibits low toxicity and high stability, while, however, being unable to elicit the action of RNase H (Wickstrom *et al.*, 1992). Phosphorothioates keep the negative charge and retain the ability to activate RNase H (Gao *et al.*, 1992). As a result, phosphorothioate oligos remain the most widely used base analogue, and are currently being tested in a number

of clinical trials (Roth and Cristiano, 1997; Wagner and Flanagan, 1997). Second generation oligonucleotides include: substituting pyrimidines at the C-5 position with 5-methyl, 5-bromo and 5-propynyluracil (Lonnberg and Vuorio, 1996) replacement of the sugar and phosphate residues with alkylamide or carbamate linkage (Crooke and Bennett, 1996; Stirchak *et al.*, 1989); 2'-O-propyl, 2'-methoxyethoxy, 2'-O-methyl, 2'-O-allyl and 2'-fluoro ribose modifications (Monia *et al.*, 1993; Wagner, 1995; Crooke and Bennett, 1996); covalent linkage of functional groups, such as cholesterol, to alter physical properties, provide ligands or provide resistance (Krieg *et al.*, 1993; Manoharan *et al.*, 1995; Crooke and Bennett, 1996); replacement of the central phosphorus with a methylene group creating a formacetal linkage (Milligan *et al.*, 1994). All of these modifications display either enhanced affinity or resistance or both, but many of them do not elicit RNase H activity. As a result, many researchers are looking towards the use of chimeric oligonucleotides, which combine segments of RNase H activating oligonucleotide, flanked by sequences which enhance nuclease resistance and target affinity (Giles and Tidd, 1992; Monia *et al.*, 1993; Giles *et al.*, 1993; Kandamilla *et al.*, 1997).

If meaningful conclusions are to be drawn from experiments with antisense oligonucleotides, the experiments must be designed carefully. Recent reviews have highlighted the problems invariably encountered from so-called non-antisense effects, where the effect seen on cell viability or protein/RNA levels is not due to the oligonucleotide acting in a specific antisense manner (Stein and Krieg, 1994; Gura, 1995; Stein, 1995; Branch, 1998). These non-antisense effects have been found to be both non-sequence and sequence specific. Non-sequence specific non-antisense effects include the binding of oligonucleotides to proteins due to their polyanion nature (Stein, 1995). In addition, the fact that RNase H can cleave sequences as short as 10 base pairs *in vivo* can lead to non-sequence specific effects. If any 10 bp segment of an oligonucleotide (of which there are 11 in a 20 bp oligo) is complementary to non-target RNA, then the non-target RNA could also be effectively cleaved (Woolf *et al.*, 1992). Sequence specific non-antisense effects include the increased affinity of sequences containing four contiguous Guanosine residues for bFGF and Laminin (a heparin binding protein) leading to perturbations of the cellular machinery (Stein, 1995). Synthetic oligonucleotides containing the CpG dinucleotide can cause murine B cells to proliferate and secrete immunoglob-

ulin, as they mimic bacterial DNA, lacking natural methylation on the internucleotide linkage (Krieg *et al.*, 1995; Stein, 1995). Phosphodiester palindromes of 6 or more bases can induce interferon α and γ production and induce natural killer cell activity Yamamoto *et al.*, 1992; Kuramoto *et al.*, 1995).

It is therefore obvious that to be certain that a given effect is due to specific antisense mechanism, rigorous controls must be employed. Initially, unique sequences should be chosen, insofar as the information databases allow, so that there is no likely cross-reaction (or as little as possible) with the other transcripts in the species being studied. If an effective antisense sequence is found, it is imperative that, at minimum, the corresponding sense sequence is shown to be inactive. However, most reviewers will now require one or more of the following additional controls: a scrambled control, a mismatch control (with one or more mismatched bases, usually in the central area of the sequence), random sequences or mismatched target controls (e.g. cell with a mutated or deleted target gene) (Stein and Krieg, 1994). It is also essential that the experimenter demonstrate a decrease in the target RNA and protein if an antisense mechanism is proposed (Stein and Krieg, 1994; Branch, 1998). However, it is likely that this may soon be insufficient as it fails to provide information about global changes in the RNA and protein populations. It may become necessary to include such rigorous testing as high-resolution two-dimensional gel electrophoresis to provide a before-and-after snap-shot of the protein populations, or differential display to allow quantitative monitoring of gene expression patterns (Branch, 1998).

Use of antisense oligonucleotides to inhibit MDR-1 expression

Over the last number of years, there have been several studies which looked at the effect of using antisense oligonucleotides to down-regulate expression of the *mdr-1* gene (See Table 1.7.1). These will be discussed in some detail, since they illustrate well some of the different approaches and achievements, as well as the limitations and problems which may be expected when applying antisense technology to investigation of drug resistance. The first such study came in 1989, carried out by Vasanthakumar and Ahmed (1989). They used a 15 base pair (bp) methylphosphonate oligonucleotide, targeted to a region containing the initiation codon of mouse *mdr-1* (see Table I), on a human

erythroleukemic cell line K562/III which had been selected for Daunorubicin resistance (107-fold compared to parent K562/S). This cell line was cross-resistant to vincristine, doxorubicin and etoposide. It also exhibited *mdr-1* amplification and increased expression of its transcripts (30-fold increase). The oligo was used at a 30 μ M concentration and was added free in the cell culture medium (which contained 10% heat-inactivated serum). After 72 h incubation time at this concentration P-170 expression was totally eliminated in the K562/III cell line. A complementary sense oligo targeted to the same region had no effect on P-170 levels. Both the antisense and sense oligos were shown to have no apparent biological effect on the parental sensitive cell line K562/S. The IC_{50} values of Daunorubicin in the K562/III cell line fell from 119-fold greater than the parent to 85-fold. There was no change in the IC_{50} values of Daunorubicin in the antisense treated parent cell line, while the sense oligos had no effect on either cell line. These results indicated the potential of using antisense against *mdr-1* to cause a down-regulation in P-170 expression and a concomitant decrease in drug-resistance. However, the antisense sequence chosen was compatible with the mouse *mdr-1* sequence and not human *mdr-1*. There were in fact three base-pair mismatches as regards the human sequence, and this should be taken into account when considering the results.

Jaroszewski *et al.* (1990), designed five different phosphorothioate oligos which were used to down regulate *mdr-1* in the human breast cancer cell line MCF-7 ADR, which was 192-fold more resistant to adriamycin than its parent cell line MCF7. One was targeted to the initiation codon region, one just 3' to the initiation codon, and three spread out within the coding region (Table I). They demonstrated the uptake and distribution of the phosphorothioate oligos in MCF-7 cells by using 35 S-labelling, showing that only 2% of the oligos were taken up from the media and, of that, 64% remained in the cytoplasm. Of the five oligos tested the most effective was the one targeted at nucleotide +18 to +32 in relation to the first base in the mRNA sequence (taken as +1). It caused a 4-fold increase in Adriamycin toxicity. However they did not present evidence of decreases in the *mdr-1* levels or give actual IC_{50} values. They also speculated on the reasons behind the variation in effect of the five oligos. They postulated that this was due to differences in the *mdr-1* mRNA secondary structure at the different sites targeted, as the mRNA is extensively folded, 62% being paired.

Table 1. Types and target sites of antisense directed against *mdr-1* mRNA

Target site	Oligo type	Cells used	Reference
-457 to -474	Phosphorothioate (PS)	LoVo/Dx,S180Dx,KbCh85	Bertram <i>et al.</i>
-20 to -1	PS oligo	CEM 6OVCR	Li <i>et al.</i>
-20 to -1	2'-O-methyl modified	CEM 6OVCR	Li <i>et al.</i>
-20 to +1	Phosphodiester	LoVo/Dx	Corrias <i>et al.</i>
-14 to +4	PS oligo	NIH 3T3	Alahari <i>et al.</i> ^a
-9 to +6	PO with PS 3' and 5' ends	Kidney primary cultures	Efferth <i>et al.</i>
-9 to +6	PS oligo	MCF-7/ADR	Jaroszewski <i>et al.</i>
-9 to +6	PS oligo	K562/VCR ₁₀₀	Sola <i>et al.</i>
-9 to +6	PS oligo	LoVo/Dx,S180Dx,KbCh85	Bertram <i>et al.</i>
-9 to +6	Methylphosphonate	K562/III	Vasanthakumar <i>et al.</i>
-9 to +9	PO oligo	LoVo/Dx	Quattrone <i>et al.</i>
-6 to +6	PO oligo	LoVo/Dx	Rivoltini <i>et al.</i>
-6 to +9	PO with PS 3' and 5' ends	SKVLB	Thierry <i>et al.</i>
-6 to +10	PS oligo	K562/ADM	Liu <i>et al.</i>
-1 to +24	PS oligo	P388/Adr	Nakashima <i>et al.</i>
+1 to +18	PS oligo	HL-60/Vinc	Cucco and Calbretta
+1 to +18	PO oligo	CHrC5	Clynes <i>et al.</i>
+1 to +20	PS oligo	P388/Doxorubicin	Hirtake <i>et al.</i>
+1 to +20	PO oligo	LoVo/Dx	Corrias <i>et al.</i>
+12 to +36	PS oligo	CEM 6OVCR	Li <i>et al.</i>
+18 to +32	PS oligo	MCF-7/ADR	Jaroszewski <i>et al.</i>
+21 to +40	PO oligo	LoVo/Dx	Corrias <i>et al.</i>
+156 to +1118	cDNA	KB 8-5	Hanchett <i>et al.</i>
+336 to +354	PS oligo	MCF-7/ADR	Jaroszewski <i>et al.</i>
+336 to +359	PS oligo	MCF-7/ADR	Jaroszewski <i>et al.</i>
+991 to +1007	PS oligo	K562/ADM	Liu <i>et al.</i>
+1152 to +1176	PS oligo	MCF-7/ADR	Jaroszewski <i>et al.</i>
+2420 to +2434	PS oligo	LoVo/Dx,S180Dx,KbCh85	Bertram <i>et al.</i>
+2990 to +3007	PS oligo	LoVo/Dx,S180Dx,KbCh85	Bertram <i>et al.</i>
+4026 to +4045	PO oligo	LoVo/Dx	Corrias <i>et al.</i>

^a Alahari *et al.* tested almost 40 different antisense sequences to various regions of the *mdr-1* transcript with different backbones and modifications. Only the most effective antisense sequence is given above.

Rivoltini *et al.* (1990) used a 12 bp phosphodiester oligo targeted to bases -6 to +6 of *mdr-1* mRNA (Table I). Human colorectal adenocarcinoma cells LoVo/Dx, which are resistant to Doxorubicin and show increased P-170 expression, were cultured for 3 days in the presence of the oligos, resulting in a reduction of P-170 to a level equal to or lower than that of the sensitive parent cell line LoVo/H. The ID₅₀ of the LoVo/Dx cells was reduced 100-fold when cultured in the presence of the antisense oligo, giving a similar effect to that obtained from the MDR modulator, verapamil.

Clynes *et al.* (1992) found that antisense, but not sense, oligodeoxynucleotides corresponding to the first 18 bases of the human *mdr-1* sequence caused

an increase in adriamycin sensitivity in the human lung squamous cell P-170 over expressing MDR line DLKP-A, and also, perhaps surprisingly in view of some sequence difference between the species, in the hamster MDR line CHrC5.

Corrias and Tonini (1992) targeted five 20 bp phosphodiester oligonucleotides (Table I) to the human *mdr-1* gene in the LoVo/Dx doxorubicin resistant cell line. They analysed stability and cellular uptake of the oligos. One of the oligos, targeted immediately 5' to the initiation codon, stood out from the others in terms of effectiveness, causing 60% of the cells to lose their resistance to doxorubicin, preventing them from forming colonies in the presence of the drug. They determined the half-life of the mRNA of *mdr-1*

to be approximately 4 h, and demonstrated that alteration of the mRNA occurred after treatment with the antisense. However, no actual IC_{50} data was given after the antisense treatment. They suggested that the other antisense oligonucleotides were ineffective due to these sequences being highly conserved among pgp and many other proteins.

In 1993, Efferth and Volm used 2x15 bp oligonucleotide targeted to bases -9 to +6 of the *mdr-1* mRNA sequence (Table I), taking in the AUG initiation codon and a Shine-Dalgarno like sequence (AGGUGG), which recognises the ribosome binding site (Efferth and Volm, 1993). One was a simple phosphodiester oligonucleotide, the other with 3 phosphorothioate bases at the 3' end. The corresponding sense sequence was used as a control. The oligos were incubated with primary cultures of kidney tumor and normal kidney from 3 different patients, for 3 days at concentrations of 1, 5 and 10 μ M. They found that the 10 μ M concentration of antisense significantly decreased P-170 levels in cells which expressed high levels of P-170 initially, while there was only a slight decrease observed for the patient with low-P-170 expressing cells. The latter patients cells also showed no inhibition of growth when the antisense was combined with vincristine or doxorubicin in a 7-day growth assay. The cells for the patients with high P-170 expression were inhibited by up to 70% by the combination of antisense and drug. The inhibitory effect was more pronounced for the 3' phosphorothioate capped oligo. This gave an early illustration of the increased efficacy of using nuclease-resistant phosphorothioate oligos when targeting *mdr-1* mRNA.

Thierry *et al.* (1993), used an unique approach to delivering the two 15 bp antisense and one 15 bp sense oligonucleotides they designed against *mdr-1* (Table I). They delivered the oligos to the human ovarian carcinoma cells SKOV3 and the multidrug resistance variant SKVLB, either free to the cells or by Minimal Volume Entrapment (MVE). Small unilamellar vesicles were made from a mixture of different lipids, which were dried by evaporation, rehydrated with phosphate-buffered saline (PBS) containing 10 μ g ml^{-1} of the oligonucleotide, vortexed vigorously and then sonicated, giving a final concentration of between 60 and 70 μ g oligo ml^{-1} lipid. 5 μ M final concentration of the oligos, which had two phosphorothioate bases at each end of a phosphodiester backbone, was used. The two antisense oligos were directed towards the AUG initiator codon-containing region (ASin) and a loop forming site located at bases +993 to +1008

from the first AUG codon (ASlp). Using a 5 μ M concentration of the ASlp oligo added free to the cell culture media, they caused a 40% reduction in P-170 expression, as measured by flow cytometry, and doxorubicin resistance. However, using MVE to deliver the oligos, they demonstrated a nearly complete inhibition of P-170 expression and a four-fold decrease in doxorubicin resistance. The lack of complete reversal of doxorubicin resistance accompanying the complete inhibition of P-170 indicated that there were other mechanisms of resistance involved in these cells. Adding free ASin oligo caused very little effect on P-170 levels or resistance, as did the freely added corresponding sense oligo. ASin added by the MVE method, however, gave a two-fold decrease in P-170 levels and a four-fold decrease in the doxorubicin resistance. This down-regulation of the *mdr-1* gene was shown to be sequence non-specific, due to the fact that the corresponding sense oligo delivered by MVE also demonstrated a significant, if not as large, decrease in P-170 expression and resistance to doxorubicin. This sequence non-specific effect was dependent on the sequence used, though, as the sense to the loop-forming region had no effect whether free or liposomally encapsulated. Thierry *et al.* postulated that bases at the 5' end of *mdr-1* coding region might be pairing intramolecularly and the sense oligo to this region might be forming a triple helix structure, thereby inhibiting mRNA processing. They proposed loop-forming regions as a more effective site for antisense targeting, and they backed this claim up by citing that another loop-targeting antisense (bases +568 to +583) was equally as effective as the one above, but they did not present data to support this. The results, therefore, demonstrated the importance of the choice of target site and also the benefits of encapsulating the oligonucleotides in liposomes. It was shown, during their work, that oligos were effectively protected from environmental degradation when encapsulated by MVE, showing no degradation after incubation for a week in 10% serum containing medium, while the free oligonucleotide was mostly degraded after 30 min.

In 1994, Quattrone *et al.*, demonstrated the use of a mixture of antisense oligos for down-regulating *mdr-1* mRNA in the 75-fold doxorubicin resistant subline (LoVo/Dx) of the human adenocarcinoma cell line LoVo (Quattrone *et al.*, 1994b). They designed three contiguous 18 bp phosphodiester oligos to attack the region from -9 to +45 from the first AUG codon (Table I), which had previously been shown to be effective (Uhlmann and Peyman, 1992). They used an equimo-

lar mixture of the three at a final concentration of $10 \mu\text{M}$ in conjunction with $5 \mu\text{g ml}^{-1}$ of the liposomal transfection reagent DOTAP. They incubated the cells in the presence of the antisense mixture for 15 days, changing the media and adding fresh oligos every 72 h. At the end of the culture, the cells were exposed to an IC_{50} value of doxorubicin (relative to the resistant and sensitive lines respectively) while maintaining the presence of the oligos. They used the three oligos together in a mixture because of a postulated synergistic inhibitor of contiguously targeted oligos (Maher and Dolnick, 1998). They demonstrated that the DOTAP used for delivery of the oligos increased their cellular uptake 25-fold and afforded protection from cytoplasmic nuclease cleavage. The 15 day incubation was employed due to the relatively long half-life of P-170 (72 h), and the fact that previous studies had only used 3 or 4 day studies, which were not deemed sufficiently long. The combination of $10 \mu\text{M}$ oligos with $5 \mu\text{g ml}^{-1}$ of DOTAP was shown to a moderate inhibitory effect on cell growth, 26 and 38% for a scrambled control oligo and the antisense oligos respectively. The decrease in cell growth when the doxorubicin was added was double the expected additive effect of growth inhibition of the antisense oligos/DOTAP mixture and the doxorubicin separately for the antisense treatment. The inhibitory effect was simply an additive for the combination of the sense oligo/DOTAP and the drug. After 5 days of the incubation, the *mdr-1* mRNA levels were shown to be significantly decreased by the antisense mixture, with no effect observed on untreated or sense treated cells. After the full 15 day incubation, the mRNA levels had decreased down to the level of the drug-sensitive parent line. As regards P-170 expression, there was no observed change after 5 days treatment with the antisense oligos, while at 15 days, there was a reduction in P-170 levels in comparison to the untreated and sense-treated cells, but not to the level of the drug-sensitive parent. They showed, however, that there was a good correlation between the *mdr-1* mRNA/P-170 levels and resistance to doxorubicin induced cytotoxicity, and postulated that the greater cytotoxic effect of the antisense/DOTAP mixture compared to the sense/DOTAP mixture, without the addition of drug, indicated that a marked and prolonged decrease in *mdr-1* gene expression could be intrinsically toxic for cells. This is a relevant consideration for possible future *in vivo* administration of *mdr-1* targeted antisense.

Hanchett and his colleagues tried a different approach by using antisense RNA transcribed from an

expression vector (Hanchett *et al.*, 1994). They used a 963 bp fragment from the *mdr-1* cDNA (bases +156 to +1118) cloned into an expression vector pH β APr-3-neo (Table I), driven by the β -actin promoter, in both the normal and reverse orientation, to give the sense and antisense molecules. They transfected the *mdr* variant, KB 8-5 (4-fold resistant to colchicine), of the human nasopharyngeal epidermoid carcinoma cell line with HeLa markers, KB 3-1, with the antisense containing plasmid, as well as the sense containing vector and the vector with no insert as controls. They isolated 16 clones from each transfection. Of the 16 selected from the antisense transfection, only 10 actually expressed the antisense RNA, and of these only 1 exhibited a decrease in *mdr-1* mRNA levels (50%) and P-170 levels (50%) as detected by Northern Blot/RNase protection assay and Western Blots respectively. This clone also displayed a corresponding decrease in colchicine resistance (50%) but was still almost 3-fold more resistant than the sensitive parent KB 3-1. Despite the large amount of effort involved in producing one successfully transfected clone, this work showed the potential of using antisense RNA as an alternative to DNA-based oligonucleotides. However, despite the fact that all cells in this population were isolated from one clone, the authors pointed out that there was a high degree of heterogeneity, with highly varying levels of P-170 expression, as demonstrated by Rhodamine 123 retention assays. Even subclones of the initial clone retained this heterogeneous phenotype, showing that it was an inherited trait of this clone. The authors went as far as performing Restriction-Fragment-Length-Polymorphism assays to prove that all phenotypically distinct subpopulations descended from the same transfected clones. This level of heterogeneity in transfected cells could be a possible deterrent to the future use of antisense RNA.

Bertram *et al.* (1995) attempted reversal of the MDR phenotype in two P-170 overexpressing variant human cell lines (LoVo/Dx^R and KBCh^R8-5) and one murine resistant cell line (S180Dx^R). They used antisense designed to four different regions in the *mdr-1* mRNA, the 5' promoter region, S-ODN1, the ATG initiation region (bases -9 to +6), S-ODN2, and two within the coding region (bases +2420 to +2434 and +2990 to +3007), S-ODN3 and S-ODN4 (Table I). They were all phosphodiester oligonucleotides with a G-C content of between 40 and 60%. They were used at a concentration of either 0.2, 2 or 5 μM , with 2 μM being the most efficient. Only a single dose of

the antisense was applied and incubated with the cells for 12 h before the addition of 10% fetal-calf serum (FCS). Cells were then further incubated for a total of 72 h before various assays were carried out. A 2 μM concentration of S-ODN2 and 3 applied to LoVo/Dx^R cells caused a decrease in [³H]thymidine incorporation of 10 and 50% respectively, with corresponding sense oligos exerting no effect. Western blots using the *mdr-1* MAb C-219 showed that S-ODN2 and 4 were unable to reduce P-170 expression in LoVo/Dx^R compared to untreated cells, while S-ODN 3 reduced the P-170 down to the level of the sensitive cells. S-ODN1 showed a less pronounced effect than S-ODN3, but still caused around a 75% reduction. Analysis of the time scale of the reduction showed that after 24 h the levels of P-170 remained unchanged, after 48 h a reduction was apparent, while after 72 h the levels of P-170 were down to the those in the sensitive parent cells. S-ODN3 was found to be most effective in the LoVo/Dx^R cell line with a 62% reduction in the ID₅₀ in the presence of doxorubicin, with the S-ODN1 only giving a reduction of 20%. In KBCh^R8-5 cells, S-ODN3 was once again more effective, decreasing the ID₅₀ by 30%, with S-ODN1 having no effect. In the murine sarcoma cell line S180Dx^R, however, S-ODN3 was ineffective, while S-ODN1 reduced the ID₅₀ in the presence of doxorubicin by 60%. The differences in effectiveness of the same oligonucleotides in different cell lines highlighted the importance of the accessibility of the target region for the antisense, which could be altered between various species and cells due to slight variations in sequence and in the processing of the mRNA. Tests comparing the effectiveness of the S-ODN3 antisense on LoVo/Dx^R cells as compared to the classic chemomodulators verapamil and tamoxifen on P-170 function, demonstrated that in this cell line at least, the antisense molecule was at least equally effective in decreasing the ID₅₀ values.

Nakashima *et al.* (1995) tested a 25 bp phosphodiester targeted to the AUG initiation codon and loop forming region (Table I) on mouse leukaemia adriamycin sensitive (P388/S) and resistant (P388/ADR) cells. The resistant cells were 100-fold and 300-fold more resistant than the sensitive cells to Vincristine and adriamycin respectively. Concentrations of oligo below 30 μM was used, due to toxicity of higher concentrations. They showed, by flow cytometry, that after 72 h the level of P-170 in the P388/ADR cells was slightly, but significantly, reduced as compared to untreated P388/ADR cells, while the sense oligo was ineffective. 15 μM of the antisense oligo caused 2-fold

more vinblastine accumulation in P388/ADR cells than the sense oligo. Correspondingly, the antisense potentiated the growth-inhibitory effect of the vinblastine, decreasing the IC₅₀ value significantly (approx. 2-fold). This reversal of resistance by the antisense was slightly more effective than verapamil on its own, and when the antisense was combined with 1 μM of verapamil, the IC value decreased approximately 4-fold.

Alahari *et al.* (1996) conducted an extensive study, analysing 32 different phosphorothioate oligonucleotides spanning almost every region of the *mdr-1* mRNA including the 5' Untranslated, AUG codon, Coding (splice junction), open reading frame (ORF), ORF and stop codon, 3' untranslated and 5' Cap (Table I). The cells used were mouse NIH3T3 fibroblasts, which had been transfected with an expression plasmid containing the human *mdr-1* cDNA (pSK1 MDR1). The antisense oligos were used at a concentration of 1 μM in the presence of 20 $\mu\text{g ml}^{-1}$ of Lipofectin, and incubated with the cells for 24 h. One oligonucleotide which stood out from the others was one overlapping the AUG start codon (AS 5995), as it caused a substantial reduction in the *mdr-1* message levels as measured by Northern blots. The other sequences tested were largely ineffective, including two other oligonucleotides which also overlapped the AUG codon. This result emphasises that slight alterations in the positioning of an antisense target can be of utmost importance in mediating antisense effects. Maximum specific reduction on *mdr-1* mRNA was observed after 24 h treatment of the cells with AS 5995, but reduction occurred only with the use of serum-free media and cationic liposomes. Multiple treatments with the AS 5995 did not cause any greater specific reduction in the *mdr-1* messenger levels than a single treatment, whereas greater cytotoxicity was observed. The reduction in the mRNA expression was found to be readily reversible after the 24 h exposure to AS 5995, with normal levels returning after 24 h if the cells were returned to complete culture medium. The treatment of the MDR 3T3 cells was concentration dependent with a slight reduction observable at 100 nM and maximum reduction (60%) at 1 μM . Higher concentrations of oligos (5–10 μM) lead to greater non-specific effects with reduced control gene β -actin message levels and cytotoxicity observed. The P-170 levels of the MDR 3T3 cells were also reduced, with the decrease being minimal after 24 h, readily detectable after 48 h and maximal after 72 h exposure to the antisense. By using a 2'-O-methyl analog of AS5995,

Alahari *et al.*, gave evidence for the role of RNase H in mediating antisense inhibition of P-170 expression. 2'-O-methyl oligonucleotides do not illicit the action of RNase H, and, as expected, this analog did not cause any reduction in P-170 levels of the MDR 3T3 cells when incubated under the same conditions as the AS 5995 oligo. Conjugation of oligonucleotides with lipophilic substituents had been reported to enhance oligonucleotide accumulation in cells and result in improved biological effects (Krieg *et al.*, 1993). They therefore synthesised a 5' cholesterol derivative of AS 5995. This analog used alone was shown to be as effective as the phosphorothioate molecule in combination with Lipofectin, causing a minimum decrease in P-170 expression of 60%. In addition, the cholesterol oligonucleotides showed less experiment-to-experiment variation than the standard phosphorothioate oligos administered with cationic lipids. By conjugating these two oligonucleotides to FITC and using flow cytometry, the authors showed that over a 2 h incubation period, the cholesterol conjugated oligo was rapidly accumulated by cells, whereas both free AS 5995 and AS 5995 complexed with Lipofectin was accumulated to a far lesser degree. After an overnight incubation, free AS 5995 still showed considerably less accumulation than the cholesterol analog, while the Lipofectin complexed AS 5995 displayed substantial but very heterogeneous cell uptake. Using confocal microscopy, these results were confirmed. In addition, while only a sub-population of the Lipofectin/antisense treated cells showed nuclear accumulation of oligo, cells treated with the cholesterol conjugate displayed uniformly extensive fluorescence in both the cytoplasm and the nucleus. These results suggested that the cholesterol conjugation enhanced the rapidity, amount and uniformity of cellular uptake of the oligonucleotide. Therefore there appears to be significant advantages in using relatively low-molecular weight cholesterol oligonucleotides compared with extremely large oligonucleotide/cationic lipid complexes.

Cucco and Calabretta (1996) used just a single 18 bp phosphorothioate antisense oligonucleotide along with sense and antisense controls (Table I). The oligos were added to sensitive HL-60 human promyelocytic cell line and a vincristine selected resistant variant, HL-60/Vinc, at a concentration of 200 $\mu\text{g ml}^{-1}$ over 4 days (80 $\mu\text{g ml}^{-1}$ on the first day, and 40 $\mu\text{g ml}^{-1}$ each subsequent day) or 360 $\mu\text{g ml}^{-1}$ over 7 days. The oligos alone had no effect on cellular proliferation. Treatment with vincristine alone

caused only a 48% inhibition at the highest concentration used (1 $\mu\text{g ml}^{-1}$). Antisense oligo in combination with vincristine treatment caused significant inhibition, 58% with 0.01 $\mu\text{g ml}^{-1}$ vincristine and 92% with 1 $\mu\text{g ml}^{-1}$ vincristine: *mdr-1* mRNA and protein levels were also reduced. There was no difference in any of these parameters with vincristine alone or vincristine with sense oligo or vincristine with scrambled oligo for sense or scrambled oligo treatment. In order to test the efficacy of this antisense oligonucleotide *in vivo*, Cucco and Calabretta, used SCID mice which were given injections of HL-60/Vinc leukaemia cells, and were subsequently treated by tail injection of antisense (1 mg/mouse/day) for 10 days in combination with vincristine (20 $\mu\text{g/mouse/day}$). Vincristine alone did not prolong the survival of the mice, when treated with sense or scrambled oligos either alone or with vincristine. Treatment with the antisense oligo alone or with vincristine was ineffective, with a median survival time of 57 days post leukaemia implant. However, the antisense and vincristine combination gave a median survival time of over 300 days. The mice were sacrificed at this time point and no trace of *c-myc* transcripts (a leukaemia cell load marker) was found by RT-PCR, indicating a lack of tumor cell presence. This evidence supports the specific mechanism of action of antisense to *mdr-1 in vivo*, leading to possibilities of future use in cancer patients.

Liu *et al.* designed two 17 bp phosphorothioate oligonucleotides complementary to ATG initiator codon region (bases -6 to +10), AS-1, and a loop forming site within the coding region (bases +991 to +1007), AS-2 (Table I) (Liu *et al.*, 1996). They tested these two antisense molecules on the human leukaemic cell line K562 and its Adriamycin resistant subline K562/Adm, which was 155-fold and 74-fold more resistant than the parent to adriamycin and vincristine respectively. Both P-170 and *mdr-1* mRNA were shown to be overexpressed in the resistant subline as compared to the sensitive parent. In the presence of adriamycin, both AS-1 and AS-2 at 10 μM concentration caused significant inhibition of K562/Adm cell proliferation after 24, 48 and 72 h incubation times. The inhibition was most prominent after 48 h, 66% for AS-1 and 72.8% for AS-2. The antisense oligos showed no inhibitory effects in the absence of adriamycin, indicating a sequence specific action of the antisense and a lack of unwanted toxicity. A control sense oligo showed no effect with or without adriamycin. Both AS-1 and AS-2, after a 48 h incubation, caused a pronounced increase in

daunorubicin accumulation from 18.2 to 67.3% and 75.2% respectively, as measured by flow cytometry. The MDR modulator Verapamil caused an increase from 18.2% to just 27.2%, indicating the much greater efficacy of the antisense oligos. Correspondingly the number of P-170 positively staining cells decreased from 71.62% for the control to 45.44% for a 10 μ M concentration of AS-1 incubated for 48 h, while the ratio of *mdr-1* mRNA expression to control β -actin expression decreased from 1.56 to 0.97.

Sola and Colombani (1996) chose to target the initiation codon of *mdr-1* using a 15 bp phosphorothioate oligonucleotide (Table I), with the corresponding sense as a control. They also used the human erythroleukemic K562 cell line and a vincristine-selected resistant subline K562/VCR₁₀₀, which had a daunorubicin IC₅₀ 40-fold higher than the parent and exhibited higher expression of P-170 (95.9% of cells) as compared to K562 cells (22.1%). The cells were incubated with 10 μ M of the antisense or sense oligos for 48 h. The antisense, but not the sense, oligo enhanced daunorubicin toxicity and rhodamine uptake in the resistant line.

Hirtake *et al.* (1997) also used just a single phosphorothioate oligonucleotide, once again targeted to the initiation codon, but this time, of murine *mdr-1* (Table I). The oligo was tested on murine multidrug resistant P388/ADR lymphoid leukaemia cell line and the parental drug sensitive P388/p. The cells were cultured for 2 days in the presence of up to 500 μ g ml⁻¹ (6.6 μ M) of the 20 bp antisense oligo and the corresponding sense. This resulted in a dose-dependent decrease in P-170 expression in the P388/ADR cell, which was reversed after a further 4 day incubation in the absence of antisense. *Mdr-1* mRNA expression was also inhibited in a dose-dependent fashion. However, no internal housekeeping gene, such as β -actin, was used as a control for these experiments, with the authors relying on an equal input of total RNA amounts for RT-PCR and an extraction of equal amounts of 18S rRNA from the AS treated cells. The adriamycin IC₅₀ on the P388/ADR cells was reduced by approximately 2 log (100-fold), giving a similar effect to that obtained with the immunosuppressant FK506, used as a positive control. No change in resistance was observed after treatment with the sense oligo. In an effort to demonstrate the *in vivo* effect of the antisense, they injected B6D2F1 mice with 1×10^6 P388/ADR leukaemia cells which causes cancerous ascites and death within 2 weeks (mean survival time 13.4 days). These mice were then treated with the antisense or

sense oligos via ip injection of 1, 3, 6 or 12 μ M of oligo/g of body weight, followed by ip injection of ADR 48 h later. With a single injection of 12 μ M antisense oligo/g body weight the mean survival time of the mice increased to approximately 24 days. When the antisense was administered twice daily for three days, the survival increased further to a mean of over 35 days. No toxicity of the oligos was observed in the mice as measured by changes in behaviour, weight gain and peripheral blood count. Although none of the antisense treated mice were actually cured, the authors emphasise that the treatment duration was short during these studies. They state that since the responses observed were dose- and time-dependent, it was reasonable to assume that longer periods of infusions and larger amount of antisense oligo would be more effective in prolonging survival or leading to a cure. However, they did not actually recover any P388/ADR cells from the mice to check for decreases in P-170 or *mdr-1* mRNA levels, and stated simply that the results suggest that this was the case. They also said that although phosphorothioate oligonucleotides are reported to be nuclease resistance *in vitro*, some results indicate that injection of these type of oligonucleotide is followed by significant degradation (90% in 24 h) (Agrawal *et al.*, 1991).

Li and his co-workers designed three antisense phosphorothioate oligonucleotides to *mdr-1* (Li *et al.*, 1997). One (1729) was just inside the coding region from bases +12 to +36, another (474) was targeted to the last 20 bases of the 5' untranslated region (-20 to -1), while the last oligo (1795) had the same sequence as 474 but with four 2'-O-methyl modified sugar linked bases at both ends (Table I). The cells which were treated were a drug resistant subline (CEM60VCR) of CCRF-CEM leukaemia cells. The oligos were added to the cells at a concentration of 1 μ M combined with 2.5 μ g ml⁻¹ of Lipofectin, and incubated for four days, with the addition of 1 ml of fresh medium containing oligonucleotide after three days. Each of the oligonucleotides decreased *mdr-1* expression by 30 to 45% as measured by antibody staining and mean relative fluorescence, while the 1795 oligo, with the DNA-RNA hybrid backbone, being significantly more effective than the other two purely phosphorothioate oligos. 2'-O-methylribonucleotides at the 3' and 5' ends of the 474 antisense gives this oligo greater *in vivo* stability and allows for increased binding affinity at the ends of the sequence, while retaining the ability to activate RNase H in the middle segment. The persistence of P-170

suppression was measured at 0, 3 and 6 days after the end of treatment. It ranged from 28–46% immediately after treatment, 20–34% 3 days after treatment and by 6 days, levels had returned to pretreatment levels. Rhodamine 123 retention was significantly increased by as little as 0.2 μM of antisense oligo, while being unaffected by control oligonucleotides. Sensitivity to vincristine was also greatly increased by antisense treatment. The LD_{50} in antisense-treated CEM60VCR cells was almost 3-fold less than in untreated cells. The induction of *mdr-1* expression through daunorubicin exposure for 24 h was examined, and was shown to be inhibited by as much as 50% by pretreating the cells for antisense oligonucleotides for 4 days. Cyclosporin induction of P-170 expression was abolished by similar pretreatment with antisense. These results show the potential of using chimeric oligonucleotide hybrids to increase the potency of antisense sequences, as opposed to using purely phosphorothioate or phosphodiester backbones. The authors also stated that as the level of resistance in CEM60VCR cells was comparable to levels of drug resistance in clinical samples, the degree of sensitisation observed (almost 3-fold) similarly might be expected to be observed in the clinic. They postulated that this level of sensitisation would be sufficient to show an improvement of the therapeutic index, and proposed a use for the antisense oligonucleotides in the clinical setting both as sensitizing agents (alone or in combination with a functional P-170 inhibitor) and as prophylactic agents during initial chemotherapy to prevent the emergence of *mdr-1* expressing clones.

Use of antisense oligonucleotides to inhibit MRP expression

Due to the relatively recent discovery of the multidrug resistance protein (MRP) as a mediator of the MDR phenotype, only a limited amount of work on the antisense modulation of MRP gene expression has so far been carried out. The first report of the use of antisense oligonucleotides for the reduction of MRP expression came from Stewart *et al.* (1996). They designed sixteen oligonucleotides (15 phosphorothioate and one 2'-O-methyl derivative) complementary to different regions along the entire length of the MRP mRNA along with one sense control oligonucleotide (see Table II). They tested these antisense sequences on a multidrug resistant MRP-overexpressing small cell lung cancer cell line, H69AR, and T5 (MRP cDNA transfected HeLa cells). Lipofectin was used

to deliver the oligonucleotides to the cells at a concentration of 5 $\mu\text{g ml}^{-1}$ for HeLa/T5 cells and 10 $\mu\text{g ml}^{-1}$ for H69AR cells. Oligonucleotide concentrations were between 0.1 and 0.5 μM as higher concentrations were found to be mildly toxic in combination with Lipofectin. The cells were incubated with the Lipofectin/oligonucleotide for 4 h, then washed and incubated in fresh serum containing medium until harvested. In some cases they were retreated with the same concentrations of Lipofectin/oligonucleotide for another 4 h after a 48 h interval. After two 4 h treatments, MRP protein levels in the H69AR cells were found to vary considerably, depending on the antisense sequence used. Two oligonucleotides were found to be reproducibly the most effective. These were termed ISIS 7597 and 7598 and were complementary to nucleotides +2107 to +2126 and +2503 to +2522 of MRP mRNA respectively. All the oligos were also screened for ability to reduce MRP mRNA levels, and after a single treatment only one was found to have no effect. This was the 2'-O-methyl oligoribonucleotide. The authors stated that as 2'-O-methyl oligonucleotides are not substrates for RNase H, a decrease in the MRP mRNA levels was not necessarily expected in the 4 h time frame of the experiment. All subsequent experiments focused on the ISIS 7597 oligonucleotide because of its efficacy in decreasing both MRP protein and mRNA levels. The other most efficacious oligo, ISIS 7598, was not chosen for further study because of its complementarity to a region highly conserved among the ABC transporter superfamily, and, as such, could potentially affect the expression of other proteins, making it less specific. Also, Stewart *et al.*, decided to carry out all subsequent tests on the HeLa/T5 cells, as resistance in this cell line was known to be solely attributable to overexpression of MRP, while the resistance in H69AR was previously shown to be multifactorial (Cole, 1992; Almquist *et al.*, 1995).

After a single treatment of T5 cells with ISIS 7597 a concentration dependent decrease in MRP mRNA was observed, with a significant decrease at 0.1 μM and virtually complete elimination of detectable MRP mRNA with 0.3 and 0.5 μM of oligo. The corresponding sense had no effect at concentration up to 0.5 μM . This reduction in MRP mRNA was transient, with maximal inhibition reached at 4 h and maintained for 24 h after treatment, but returning to 70 and 100% of those in untreated controls by 48 and 72 h, respectively. 0.5 μM of ISIS 7597 decreased MRP protein levels transiently and maximally to 30% of control

Table 2. Types and target sites of antisense targeted to MRP mRNA

Target site	Oligo type	Cells used	Reference
-194 to -176	Phosphorothioate	H69AR, HeLa/T5	Stewart <i>et al.</i> (1996)
-106 to -87	Phosphorothioate	H69AR, HeLa/T5	Stewart <i>et al.</i> (1996)
-20 to -2	Phosphorothioate	H69AR, HeLa/T5	Stewart <i>et al.</i> (1996)
+1 to +16	Phosphorothioate	H69AR, HeLa/T5	Stewart <i>et al.</i> (1996)
+19 to +38	Phosphorothioate	H69AR, HeLa/T5	Stewart <i>et al.</i> (1996)
+106 to +125	Phosphorothioate	H69AR, HeLa/T5	Stewart <i>et al.</i> (1996)
+1911 to +1930	Phosphorothioate	H69AR, HeLa/T5	Stewart <i>et al.</i> (1996)
+1911 to +1930	2'-O-propyl/PS chimera	HeLa/T5	Canitrot <i>et al.</i> (1996)
+1911 to +1930	2'-O-fluoro/PS chimera	HeLa/T5	Canitrot <i>et al.</i> (1996)
+2307 to +2326	Phosphorothioate	H69AR, HeLa/T5	Stewart <i>et al.</i> (1996)
+3328 to +3347	Phosphorothioate	H69AR, HeLa/T5	Stewart <i>et al.</i> (1996)
+4526 to +4545	Phosphorothioate	H69AR, HeLa/T5	Stewart <i>et al.</i> (1996)
+4579 to +4590	Phosphorothioate	H69AR, HeLa/T5	Stewart <i>et al.</i> (1996)
+4616 to +4635	Phosphorothioate	H69AR, HeLa/T5	Stewart <i>et al.</i> (1996)
+4639 to +4658	Phosphorothioate	H69AR, HeLa/T5	Stewart <i>et al.</i> (1996)
+4742 to +4761	Phosphorothioate	H69AR, HeLa/T5	Stewart <i>et al.</i> (1996)
+4789 to +4808	Phosphorothioate	H69AR, HeLa/T5	Stewart <i>et al.</i> (1996)

levels after 48 h. This is consistent with the previously determined half life of MRP protein and the kinetics with which MRP mRNA levels are depleted and restored. When a double treatment with 0.5 μ m of ISIS 7597 was used, the levels of MRP mRNA 24 h after the second treatment were approximately 10% of those in control cells. 24 h later again, the mRNA levels had returned to 30% of the controls. A 0.3 μ m concentration of the oligonucleotide caused equal but more transient reduction. MRP protein levels were also decreased by 90% 24 h after the second treatment, and this reduction was maintained for an additional 24 h. Thus, a double treatment of HeLa/T5 cells with ISIS 7597 was able to extend significantly the response at the mRNA level to at least 3 days and to decrease MRP protein levels by 90%. The authors also demonstrated the role of RNase H in the reduction of MRP mRNA levels. By using two DNA probes for Northern blots corresponding to regions in the 5' and 3' coding ends of the mRNA, they were able to detect the oligonucleotide induced cleavage fragments of MRP mRNA in whole cells.

Stewart *et al.*, proposed that variations in secondary structure at different sites within the MRP mRNA may have been the cause of differences in efficacy of the various oligonucleotides tested. The AUG start site, as seen above, has been targeted in many studies because of the proposed accessibil-

ity of the this sequence. However, this group found that oligos complementary to the coding region to be the most effective. These results indicate the importance of evaluating the activity of a number of oligonucleotides complementary to different regions of a given mRNA target rather than testing oligonucleotides directed against a single site. They also postulated that, variations in the doubling time of cells had an effect on the transiency of the antisense effect in these cells. They observed that, of the two cell lines they tested, inhibition was more prolonged in H69AR cells, which have a doubling time significantly longer than HeLa/T5 cells. They suggested that the rapidity with which MRP mRNA returns to normal levels is influenced by the decrease in intracellular concentration of oligodeoxynucleotide during cell division.

Many of the same group were involved in the second report of MRP directed antisense by Canitrot *et al.* (1996). The work presented was a continuation of that described above by Stewart *et al.* (1996). They used the same ISIS 7597 sequence with proven efficacy and made 2'-modifications in an attempt to improve this efficacy further. There were two oligonucleotides with different numbers of 2'-O-Propyl modified bases at both ends, while there were three oligos with 2'-O-F modification, either at both or just at single ends (Table II). They were attempting to determine whether or not the 2'-O-modified

chimeric analogs containing various 2'-O-deoxy gaps were more effective at reducing MRP mRNA expression than the 2'-O-deoxyphosphorothioate ISIS 7597. The cells used in the assays were once again the HeLa/T5 cells derived by stable transfection of the MRP cDNA expression vector, pRc/CMV-MRP1. In the first set of experiments the effect of the modified chimeric oligos on viability of the cells was examined. The results indicated that the 2'-O-Fluoro/2'-O-deoxy oligos were considerably more toxic than the 2'-O-propyl/2'-O-deoxy oligos and the basic ISIS 7597. The Fluoro modified oligos were toxic at concentrations greater than 0.1 μM whereas the propyl modified oligos were not toxic up to 0.5 μM . The T5 cells were then exposed to 0.5 μM of the oligos for 4 h and RNA and protein samples were isolated at various times thereafter. All the modified oligos markedly but transiently reduced both the MRP protein and mRNA levels, by between 60 and 70% for both. There was complete restoration of the mRNA levels after 24 h. Once again, the role of RNase H in the decrease in MRP mRNA levels was demonstrated by using 3' and 5' Northern Blot probes to identify mRNA cleavage products. The bands obtained were of size 2.3 and 3.2 kb, which would be expected from the 5.5 kb MRP mRNA. Similar results were obtained for both fluoro and propyl modified oligos. To assess whether the reduction in MRP protein and mRNA levels was associated with enhanced chemosensitivity, cells were treated with 0.5 μM of the 2'-O-propyl modified oligos for 4 h and then exposed to various concentrations of doxorubicin. The results indicated that, as with ISIS 7597, these oligos caused a significant but only partial reversal of MRP-mediated resistance. The 2'-O-fluoro modified oligos were not tested because of their inherent toxicity. So, in summary the 2'-O-modified chimeric oligonucleotides were equally, but not more, effective as the 2'-O-deoxyphosphorothioate oligonucleotides, with the Fluoro modification being increasingly cytotoxic.

Antisense oligonucleotides directed against apoptosis related genes

Several studies have been carried out to date using antisense oligodeoxynucleotides against oncogenes and proteins involved in the modulation of apoptosis. Some examples are discussed below.

Casalini *et al.* (1997) reported a study on the inhibition of tumorigenicity in a lung adenocarcinoma cell line, Calu3, which overexpresses the c-erbB-2

oncogene. The results found suggest a correlation between c-erbB-2 overexpression and tumorigenicity. Antisense cDNA constructs encompassing different regions of the c-erbB-2 gene resulted in a corresponding reduction in RNA and protein levels. Similar studies have been carried out by Colomer *et al.* (1994), Liu *et al.* (1996) and Vaughn *et al.* (1996).

Tari *et al.* (1994) targeted CML cells expressing the p210 bcr-abl protein with methylphosphonate oligodeoxynucleotides incorporated in liposomes. The oligonucleotides (at 1 μM concentrations) targeted to the breakpoint junctions of the bcr-abl mRNA achieved fifty percent growth inhibition. The results were found to be specific for CML cells. A novel and more selective treatment of CML was attempted by Skorski *et al.* (1993) when they treated a 1:1 mixture of bcr-abl cells and normal bone marrow cells with mafosfamide (at a less toxic dose than that used for purging of bcr-abl expressing cells from marrows) and bcr-abl antisense oligodeoxynucleotides. The results indicated that this combination of a conventional chemotherapeutic drug and a tumour-specific antisense oligodeoxynucleotide was highly effective in killing leukemic cells and in sparing a much higher normal number of progenitor cells as compared with high-dose mafosfamide treatment. Further studies carried out by the same group (Skorski *et al.*, 1997) revealed that this combination treatment was associated with increased induction of apoptosis and that the cellular uptake of bcr-abl antisense oligodeoxynucleotides was increased twofold to six-fold by prior treatment with mafosfamide.

Seimiya *et al.* (1997) showed that reduction of JNK1 protein levels (using JNK1 antisense, but not sense, oligodeoxynucleotides) in the myeloid leukaemia cell line, U937, reduced the rate of apoptosis induced by etoposide or camptothecin; there was a corresponding reduction in caspase-3 activation.

Los *et al.* (1997) demonstrated that tumour cells resistant to CD95 (APO-1/Fas)-mediated apoptosis were cross-resistant to apoptosis-induced by anticancer drugs such as doxorubicin and methotrexate. Following drug treatment, a strong increase of caspase activity was found that preceded cell death. Antisense strategies targeted at caspase-1 and -3 strongly inhibited drug-induced apoptosis. CD95-resistant cell lines that failed to activate caspases upon CD95 triggering were found to be cross-resistant to drug-mediated apoptosis. The authors concluded that the identification of defects in caspase activation may provide mole-

cular targets to overcome drug resistance in tumour cells.

Walker and co-workers (1996) found that following a single-dose of 1 mg kg^{-1} cisplatin chemotherapy rat c-myc expression was elevated two-fold and was sustained for up to 7 days. Antisense approaches targeted at the c-myc gene reduced cell growth and the authors concluded that antisense therapy directed at c-myc in combination with cisplatin may achieve therapeutic efficacies *in vivo*. Van Waardenburg *et al.* (1997) studied the effect of c-myc antisense oligodeoxynucleotides in a c-myc over-expressing cisplatin-resistant small cell lung cancer cell line and demonstrated that a reduction in c-myc RNA levels resulted in a corresponding inhibition of growth (50% during the first 48 h), an increase in apoptosis (2-fold within 24 h) and an increase (1.4 fold) in cisplatin sensitivity without alteration to doxorubicin or vincristine sensitivities. A previous study by the same group (Van Waardenburg *et al.*, 1996) demonstrated that pre-incubation with $15 \mu\text{M}$ antisense deoxynucleotides targeted at the c-myc gene induced growth inhibition in the SCLC cell line, GLC4 and cisplatin and adriamycin resistant variants of the same cell line. Cisplatin sensitivity was increased in the cisplatin-resistant variant but not in the parental GLC4 cell line. No effect on adriamycin sensitivity was seen. The effect on cisplatin resistance was not found to be mediated by changes in cell cycle distribution. Mizutani *et al.* (1994) also found in a study of urinary bladder tumour cell lines that the effect of c-myc antisense oligodeoxynucleotides on chemotherapeutic drug sensitivities was cisplatin specific. A combination treatment of c-myc antisense phosphorothioate oligodeoxynucleotides and cisplatin was also found to enhance growth inhibition of the human colon carcinoma cell line LoVo Dx both *in vitro* and in nude mice bearing LoVo Dx solid tumours. Similar results were found by Mizushima *et al.* (1995) for combination treatment of N-myc antisense oligodeoxynucleotides and cisplatin cytotoxicity. Leonetti *et al.* (1996) also found that treatment of human melanoma cells with c-myc antisense phosphorothioate oligodeoxynucleotides inhibited the growth of these cell lines and was associated with the induction of apoptosis.

Gallagher *et al.* (1997) demonstrated, using antisense deoxynucleotides targeted at the p53 gene, that inactivation of the p53 protein resulted in resistance to cisplatin in the ovarian A2780 cell line.

To examine the relevance of Bcl-2 to drug resistance, Kitada *et al.* (1994) used anti-sense tech-

niques to reduce the levels of steady-state Bcl-2 protein levels in t(14;18) containing human lymphoma cell lines. Treatment of the t(14;18) containing human lymphoma line, SU-DHL-4, with 18-mer synthetic bcl-2 antisense oligodeoxynucleotides produced sequence-specific reductions in the relative levels of bcl-2 mRNA within one day; a corresponding reduction in protein levels took longer (3 days). The reduction in Bcl-2 protein levels in these cells did not accelerate the rate of cell death but rather it rendered cells more prone to apoptosis caused by an additional stress such as treatment with chemotherapeutic drugs (e.g. methotrexate and arabinoside C). MCF-7 cells (oestrogen receptor positive cell line) express bcl-2 mRNA when grown in the presence of oestrogen. Oestrogen depletion was also found to sensitise MCF-7 cells to the cytotoxic effects of adriamycin (Teixeira *et al.*, 1995). The authors found that in the presence of oestrogen, MCF-7 cells expressing bcl-2 antisense transcripts were partially sensitised to the effects of adriamycin. The authors conclude that the results presented support the theory that some anti-oestrogens increase the sensitivity of breast cancer cells to adriamycin by decreasing cellular levels of Bcl-2. A number of other studies have been published which describe the use of bcl-2 antisense deoxynucleotides as a means of down-regulating Bcl-2 expression and growth retardation in tumour cells (Capaccioli *et al.*, 1996; Harnois *et al.*, 1997; Ziegler *et al.*, 1997). The use of antisense oligodeoxynucleotides to bax mRNA in rat sympathetic neurons in culture has also been described (Gillardon *et al.*, 1996). Treatment with bax antisense oligos caused a significant reduction of Bax protein which was found to promote neuronal survival in the presence of insufficient growth factors. Webb *et al.* (1997) have described the first study of Bcl-2 antisense therapy in human beings. In this study, a daily subcutaneous infusion of 18-base, fully phosphorothioated antisense oligonucleotide was administered for 2 weeks to nine patients who had Bcl-2 positive relapsed non-Hodgkin lymphoma. No side effects of the treatment were observed and the antisense therapy led to an improvement in symptoms, objective biochemical and radiological evidence of tumour response, and down-regulation of the Bcl-2 protein in some patients.

Antisense approaches in the study of MDR: conclusion

The results from the various authors cited above, demonstrate clearly the potential usefulness of us-

ing antisense oligonucleotides, to effectively down-regulate the expression of MDR-related genes. It appears that the use of liposomal carriers for the transfer of the antisense oligos into the cell can greatly enhance the efficacy and prolonged activity of the oligos, through efficient delivery to the intracytoplasmic and nuclear regions and affording protection from cellular nucleases. The conjugation of oligonucleotides to lipophilic substituents also appears to be a very effective means of delivering the antisense directly to the nucleus. However, it is also apparent that a large amount of work remains to be done in this area. In almost all of the studies carried out above, full reversion of multiple drug resistance was not achieved. In many cases, this is due to the MDR phenomenon being multifactorial, with a combination of proteins causing increased cytotoxic drug resistance, so that reducing the expression of any one individual gene will not eliminate the MDR phenotype. Obviously, tumour-specific delivery is another important challenge to be overcome in bringing the technology to routine application in cancer therapy.

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