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Oligonucleotide Modulation of Multidrug Resistance

D.Y. Bouffard,[1] T. Ohkawa,[1] H. Kijima,[1,*] A. Irie,[1] T. Suzuki,[1] L.D. Curcio,[2] P. S. Holm,[1] A. Sassani[1] and K.J. Scanlon[1]

[1]Section of Biochemical Pharmacology, Department of Medical Oncology, Montana Building; and [2] Department of General and Oncologic Surgery, City of Hope National Medical Center, 1500 E. Duarte Road, Duarte, California 91010, U.S.A.

INTRODUCTION: MULTIDRUG RESISTANCE CHARACTERISTICS

TUMOUR CELLS often obtain cross-resistance to structurally and functionally unrelated lipophilic drugs. This phenomenon, termed multidrug resistance (MDR), is one of the major obstacles for the chemotherapeutic treatment of malignancies. It now appears that there may be a class of genes (MDR-like proteins) involved in maintaining the MDR phenotype, and these genes may act alone or in combination to maintain the drug resistant phenotype. MDR genes may help to explain the often seen difficulties in reversing the MDR phenotype.

In 1976, Juliano and Ling isolated a 170 kDa protein named P-glycoprotein, from the plasma membranes of Chinese hamster ovary cells displaying the MDR phenotype [1]. This P-glycoprotein is found to be overexpressed in a large number of multidrug resistant mammalian cells [2, 3]. Structurally, this protein consists of 1280 amino acids which are encoded by the multidrug resistance gene (*MDR1*) [4–7]. The MDR-related P-glycoprotein consists of two homologous parts. Each part of the protein includes a hydrophobic region with six predicted transmembrane domains and a hydrophilic region that forms a pore-like structure [8]. This protein contains two cytoplasmic ATP-binding sites [9] and possesses drug-binding properties [10]. The glycoprotein functions as an ATP-dependent drug efflux pump serving to decrease the accumulation of structurally unrelated cytotoxic agents in the intracellular milieu [11].

A non-P-glycoprotein-mediated MDR phenotype has recently been shown in several cell lines such as the human small cell lung carcinoma, H69AR [12] and the human leukaemia cell line, HL60/Adr [13, 14]. This MDR phenotype is characterised by the overexpression of a multidrug resistance-associated protein (MRP), which is a 190 kDa ATP-binding glycoprotein consisting of 1531 amino acids [15, 16]. MRP is thought to function as an ATP-dependent drug-efflux pump in plasma membrane similar to the *MDR1* P-glycoprotein [16, 17]. More recently, another new MDR phenotype was reported in a non-small cell lung carcinoma cell line SW-1573/2R120 [18]. This new MDR phenotype overexpresses a lung resistance-related protein (LRP), which is a 110 kDa protein consisting of 896 amino acids [18, 19]. This new protein, encoded by the *LRP* gene is located in the proximity of the *MRP* gene on chromosome 16, and also shows an ATP-dependent drug accumulation defect [18, 20]. If new agents or strategies are to overcome this MDR phenotype, a better understanding of the molecular biology of these genes, whether they act alone or in collaboration, will be required for effective therapy.

SIGNAL TRANSDUCTION RELATED TO THE MDR PHENOTYPE

Cell growth and differentiation can be modulated by signal transduction pathways. Several kinds of cancer chemotherapeutic agents have been shown to possess the ability to disrupt these signal transduction pathways, and induce multidrug resistance by several mechanisms [21, 22]. The relationship between the signal transduction pathway and the activated P-glycoprotein is shown in Figure 1. Activation of the signal transduction pathway can stimulate protein kinase C (PKC) which in turn can phosphorylate and therefore activate the P-glycoprotein [23, 24]. Activation of *RAS* and PKC has also been shown to stimulate *JUN* and *FOS* expression [25, 26]. A heterodimeric complex of several different proteins, consisting of the *JUN* and *FOS* families, forms the activator protein-1 (AP-1). AP-1 responsive genes are important in DNA synthesis, DNA repair and drug detoxification. The promoter/enhancer element of the *MDR1* gene contains the AP-1 binding site sequence. Since the transcription efficiency of the *MDR1* gene appears to be regulated by AP-1 [27, 28], the activation of *FOS* and *JUN* may lead to increased expression of the *MDR1* gene.

Increased expression of the *C-FOS* oncogene is found associated with several drug resistance mechanisms, and several of these drugs are not associated with the MDR family [29, 30]. *FOS* is thought to mediate its effects through transcriptional activation after interaction with the Jun protein to form the AP-1. Therefore, overexpression of *FOS* may cause the MDR phenotype by modulation of *MDR1* gene expression. A ribozyme that downregulates the *C-FOS* mRNA has been shown to reverse the resistance to chemotherapeutic agents, and regulated not only *C-FOS* expression but also the

Correspondence to K.J. Scanlon currently at the Cancer Research Department, Berlex Biosciences, 15049 San Pablo Avenue, P.O. Box 4099, Richmond, California 94804-0099, U.S.A.
*Present address: Tokai University School of Medicine, Department of Pathology, Bohseidai, Isehara, Kanagawa 259-11, Japan.

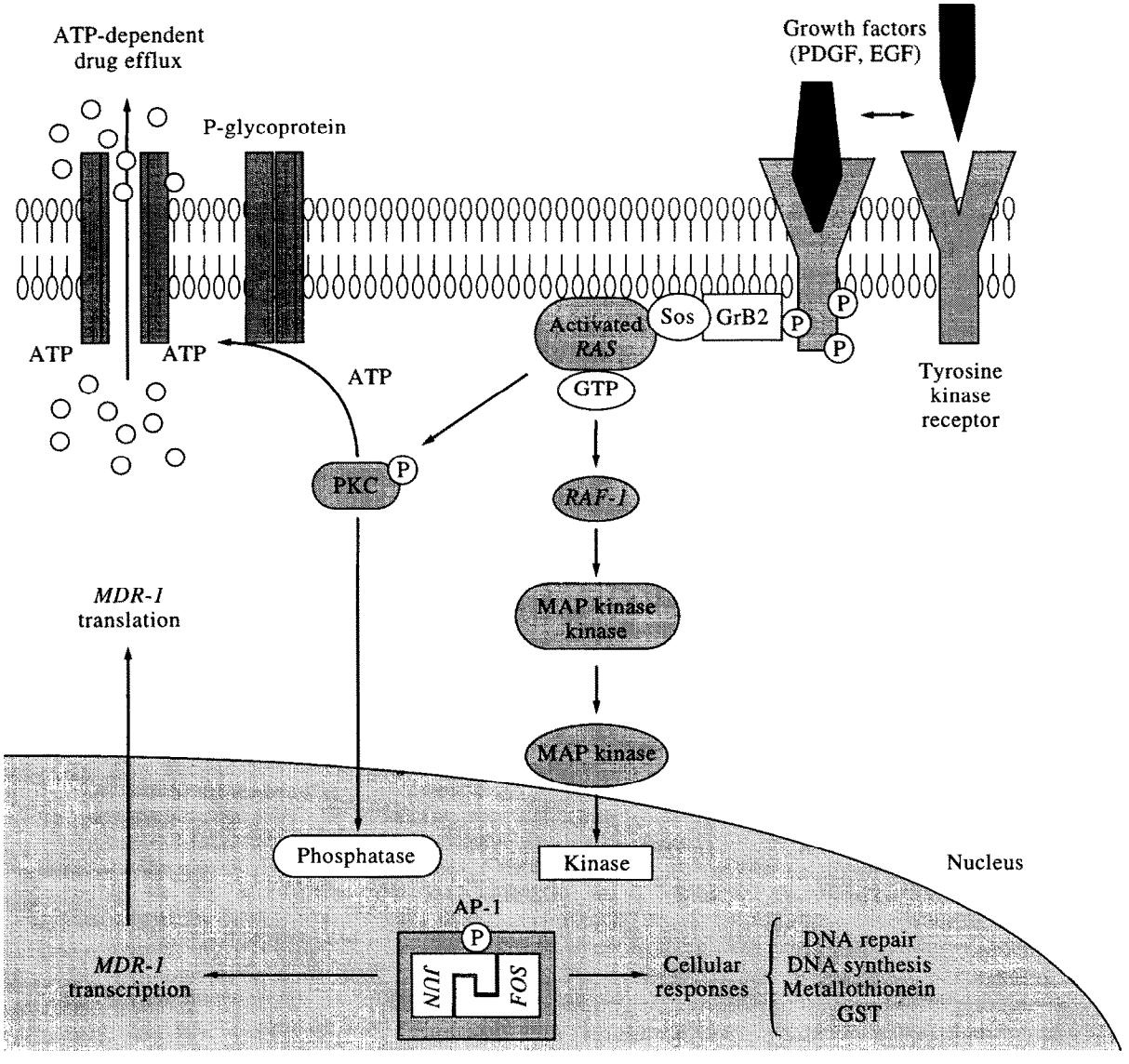


Figure 1. Signal transduction pathway associated with the P-glycoprotein-mediated MDR phenotype.

expression of *MDR1*, *C-JUN*, topoisomerase I and mutant *TP53* [29]. The suppression of *C-FOS* expression may reverse drug resistance by several mechanisms involving DNA synthesis and repair. Therefore, components of the signal transduction pathway could be important factors for reversing the drug resistant phenotype.

Several types of strategies have been utilised to modulate the MDR phenotype both *in vivo* and *in vitro*. Inhibition of the P-glycoprotein has been achieved with the use of specific drugs, such as the calcium channel blocker, verapamil, and the immunosuppressive agent, cyclosporin A. Verapamil-induced reversal of the P-glycoprotein function, both *in vitro* and *in vivo*, was first demonstrated in a vincristine-resistant P388 leukaemia model [31]. In these experiments, verapamil was shown to have the ability to enhance the cytotoxicity of vincristine and vinblastine in this resistant cell line. Since then, other publications have shown the efficacy of verapamil to modulate the MDR phenotype, both in cell culture and clinically [32–34]. However, the toxicity associated with verapamil has limited its clinical use and has lead to new strategies to reverse the P-glycoprotein function.

Among other drugs used against P-glycoprotein, cyclosporin A is most effective in haematopoietic cancer cells [35, 36]. However, due to its immunosuppressive and nephrotoxic effects, cyclosporin A does not represent a good candidate for clinical settings. New quinoline derivatives MS-209 and MS-073 have also shown their ability to modulate the MDR phenotype, but the toxicity of these compounds needs further investigation in order to evaluate their potential utilisation *in vivo* [37, 38]. Recently, a quinoline antimicrobial agent, difloxacin, has been shown to reverse *in vitro* the MRP multidrug resistance phenotype in a subset of HL-60 cells resistant to doxorubicin [39]. The difloxacin chemosensitising effect was observed with clinically achievable concentrations, and may represent a potential drug for the reversal of MRP multidrug resistance *in vivo*, although its toxicity needs evaluation in patients.

Antibodies have also demonstrated their efficacy to alter the MDR phenotype in cultured cell lines. Tong and colleagues [40] found that the combination of a plasma cell reactive monoclonal antibody and a P-glycoprotein-reactive monoclonal antibody MRK-16 could co-operate to eliminate

chemoresistant multiple myeloma clonogenic cells while affecting partially sensitive cells. Other strategies used to reverse the multidrug resistance phenotype have included oligodeoxynucleotides (ODNs) and hammerhead ribozymes, which have been shown to effectively suppress P-glycoprotein function. The following section will discuss in more details the rationale of ODN-mediated targeting of the *MDR1* gene.

ANTISENSE STRATEGIES FOR MODULATING THE MDR PHENOTYPE

In recent years, researchers have extensively studied the molecular basis of oligonucleotides and defined them as specific modulators of gene expression. Current studies have demonstrated the potential of these oligonucleotides as future therapeutic agents for overcoming the MDR phenotype in cancer. Oligonucleotide-mediated strategies include antisense oligodeoxynucleotides (ODNs) and ribozymes (catalytic RNAs). This section will focus on ODN-mediated reversal of the MDR phenotype.

Antisense ODN strategies emerged when early investigations showed that bacteria regulate gene replication and transcription by the elaboration of small complementary or antisense RNA molecules [41]. Zamecnik and Stephenson [42] were also able to demonstrate that the use of a specific ODN, complementary to the Rous sarcoma virus 35S RNA, could inhibit virus replication and cell transformation. Since then, other investigators have developed oligonucleotide-mediated gene modulation using single-strand DNA or RNA to other targets. Antisense oligonucleotides have the ability to form complementary double-helix structures with their target mRNAs, and inhibit their translation [43]. Several mechanisms of ODNs-mediated gene modulation have been published [44–46]. One model involves the conventional binding of ODNs directly to their target mRNAs producing an inhibition of their translation by the ribosomes. Recently, many researchers have clarified that RNase H activity plays a most important role in downregulating the expression of the target gene [47, 48]. RNase H is an unbiquitous ribonuclease that specifically digests the RNA strand of an RNA/DNA heteroduplex [49]. In antisense strategies, specific ODNs form a complementary duplex with its target mRNA; the mRNAs of the RNA/DNA duplex are then digested by RNase H. This mechanism is catalytic and has the advantage that one antisense ODN molecule can inactivate many target mRNAs.

Chemical substitutions of the natural DNA structure of ODNs can increase their stability [50, 51]. Chemical modifications at the internucleotide phosphodiester linkage is important because this linkage is sensitive to nuclease of the extracellular matrix. One example of such modifications is a phosphorothioate ODN [52]. Phosphorothioate-modified ODNs keep their relative charge and act as substrates for RNase H at concentrations lower than that of their complementary RNAs [53]. In addition, a methylphosphonate ODN has been shown to exhibit low toxicity and high stability in mice, but without affecting the mRNA level of its target [54]. However, its low activity might be due to the fact that it does not act as a substrate for RNase H activity [55]. Methylphosphonate ODNs may directly inhibit gene translation [56]. Although both phosphorothioate and methylphosphonate ODNs have some limitations, their resistance to nucleases makes them attractive for *in vivo* experiments. In fact, both of these modified ODNs are nearing clinical trials [52]. Several groups have demonstrated antisense-related modulation of the *MDR1* gene in human cancer cells (Table 1, Figure 2, [57–64, 89]). Vasanthakumar and Ahmed [57] were the first to demonstrate that a 15-base ODN (nucleotide −9 to +6) completely inhibited P-glycoprotein synthesis in the K562/III erythroleukaemia cells resistant to daunorubicin. Other groups have also used a similar sequence of antisense ODN [59, 61, 64]. However, in these experiments the sequence used for the 15-base ODN was compatible with the mouse *mdr1* gene, and contained three mismatches relative to the corresponding fragment of the human *MDR1* gene. Therefore, these results cannot be regarded as a classical antisense effect.

Different ODN sequences were designed to target *MDR1* gene expression. The majority of these targets were sequences located around the mRNA AUG initiation codon (Figure 1, Table 1). Several investigators evaluated different ODN sequences targeting *MDR1* [51, 58, 60]. Proposed mechanisms to explain the fact that different ODNs had varying effects on *MDR1* gene modulation were: (1) the extensively folded secondary structure of the *MDR1* mRNA was responsible for varying effects (62% of all *MDR1* mRNA is paired) [58]; (2) the sequences of ineffective ODNs are highly conserved not only in the P-glycoprotein, but also among many proteins [60]; (3) choice of the cell line may affect the overall effect of the ODNs [61].

Based on chemical modification studies, many investigators have demonstrated *MDR1* gene modulation using phosphorothioate ODNs [58, 59, 61, 62], while a few groups used unmodified ODNs [63] or methylphosphonate ODNs [57]. Comparison between unmodified and modified ODNs targeting the human *MDR1* gene has not been extensively studied. Efferth and Volm [59] reported that a phosphorothioate ODN was more effective in inhibiting cell growth when compared with its natural ODN. Another group compared the effects of antisense ODN with the classical modulator verapamil [64]. A three-day incubation with this ODN reduced the ID_{50} of the LoVo/Dx colorectal carcinoma cells, and a similar effect was obtained with the classical modulator, verapamil.

Some reports studied different delivery systems for antisense ODNs directed against the *MDR1* gene. Quattrone and coworkers [63] demonstrated that unmodified ODNs carried by a synthetic cationic lipid vector reduced the level of the *MDR1* mRNA and P-glycoprotein by approximately 50%. Thierry and coworkers [62] reported that the cellular uptake and intracellular release of ODNs were facilitated when the ODNs were delivered by liposomal encapsulation. The SKVLB ovarian carcinoma cells treated with liposome-encapsulated ODNs exhibited a nearly complete inhibition of P-glycoprotein expression and a 4-fold decrease in doxorubicin resistance. In contrast, SKVLB cells exposed to free ODNS showed a 40% inhibition of P-glycoprotein expression and doxorubicin resistance.

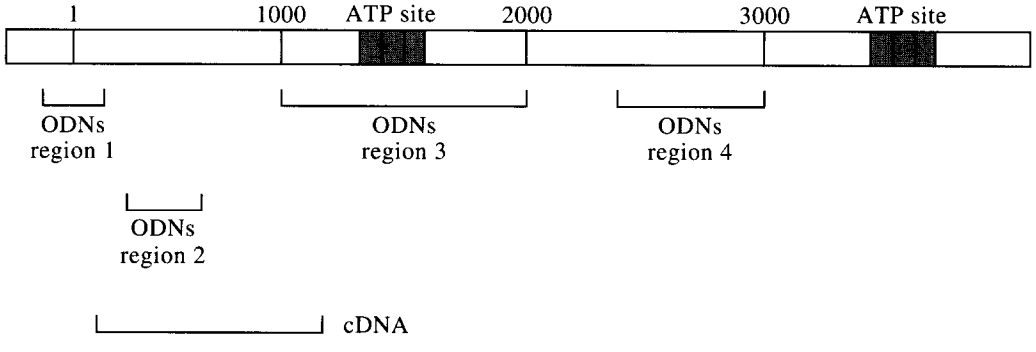
Hanchett and colleagues [65] reported in their unique study that an antisense *MDR1* expression vector could reverse the MDR phenotype in the KB 8-5 nasopharyngeal epidermoid carcinoma cells resistant to colchicine. The KB 8-5 cells were transfected with the antisense *MDR1* RNA expression vector pHβApr-3-neo, including 963 bases of the *MDR1* cDNA, and driven by the human β-actin promoter. One transfected clone was shown to have a 50% decrease of the *MDR1* mRNA and P-glycoprotein level.

The studies mentioned above have demonstrated that ODNs are capable of inhibiting the MDR phenotype specifi-

Table 1. *Antisense targets for the* MDR1 *transcript*

ODN Region	Target site	Cell type	[Ref.]
1	−20 to −1	LoVo	[60]
1	−10 to +18	MCF-7	[58]
1	−9 to +6	K562	[57]
1	−9 to +6	Primary	[59]
1	−9 to +6	LoVo	[61]
1	−9 to +6	LoVo	[64]
1	−9 to +9	LoVo	[63]
1	−9 to +9	SKVLD	[62]
1	−1 to +24	P388	[89]
1	+1 to +20	LoVo	[60]
1	+10 to +27	LoVo	[63]
1	+18 to +32	MCF-7	[58]
1	+21 to +40	LoVo	[60]
1	+28 to +45	LoVo	[63]
2	+336 to +354	MCF-7	[58]
2	+336 to +359	MCF-7	[58]
3	+993 to +1008	SKVLD	[62]
3	+1152 to +1176	MCF-7	[58]
3	+2066 to +2085	LoVo	[60]
4	+2420 to +2434	LoVo	[61]
4	+2990 to +3007	LoVo	[61]
5	+4026 to +4045	LoVo	[60]

(a) Antisense targets for the *MDR*-1 transcript



(b) Ribozyme targets for the *MDR*-1 transcript

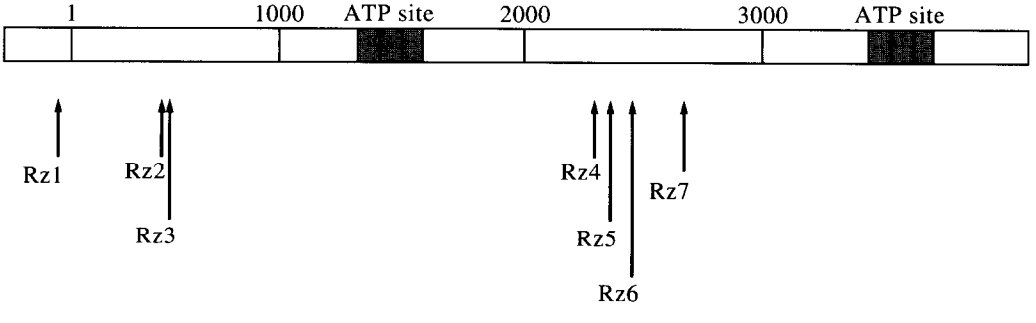


Figure 2. Targeted regions of the *MDR1* transcript by (a) ODNs and (b) ribozymes.

Table 2. Ribozyme targets for the MDR *transcript*

Ribozyme number	Cleavage site	Cell type	[Ref.]
Rz1	−4	PXF118	[79]
Rz2	+537	Molt-3	[81,82]
Rz3	+538	Molt-3	[81,82]
Rz4	+2408	Test tube	[88]
Rz5	+2429	LoVo	[61]
Rz6a	+2440	LoVo	[61]
Rz6b	+2440	Test tube	[88]
Rz7a	+2639	A2780	[85]
Rz7b	+2639	EPP85-181	[87]

cally. These data suggest that antisense ODN-mediated therapy could become a useful clinical approach for reversing the MDR phenotype in the future. However, the most critical issues for their future therapeutic utility rely on their stability and effective delivery to the target cancer cells.

RIBOZYME STRATEGIES FOR MODULATING THE MDR PHENOTYPE

The central dogma that only enzymes have a catalytic potential has changed with the discovery of small catalytic RNA molecules (i.e. ribozymes). The first demonstration that RNA molecules possess catalytic activity was reported by Cech and coworkers [66] and Altman [67]. Subsequently, other studies have demonstrated the presence of ribozymes in plant virus-like particles called viroids [68]. Ribozymes have been recently classified into six groups: (1) ribozymes derived from self-splicing of *Tetrahymena* Group I introns [66, 69]; (2) RNA components of RNase P [70]; (3) hammerhead ribozymes [71]; (4) hairpin ribozymes [72]; (5) genomic and anti-genomic RNase of hepatitis δ virus [73]; and (6) RNA transcripts of mitochondrial DNA plasmid of *Neurospora* [74]. Because of its simple self-cleavage domain of its small size, ribozymes, especially hammerhead ribozymes, have found utility as a potential therapeutic agent [75–77]. This section will focus on the use of ribozymes to modulate genes which are associated with the multidrug resistance phenotype, such as *MDR1* and *C-FOS*.

A major advantage of using ribozymes compared with conventional antisense ODN-mediated strategy is its specific catalytic potential. One molecule of the designed hammerhead ribozyme can cleave several of the chosen target mRNA in *trans*. This implies that, theoretically, lower concentrations are required. After the cleavage reaction, the substrate is accessible by RNases; this step guarantees its permanent inactivation. The advantages of ribozymes in comparison with ODNs have already been confirmed in several experiments [78, 79]. Since it is possible to design RNA/DNA chimeric ribozymes [80], which can increase the rate of the cleavage reaction, the activity of RNase H could also become important in downregulating the target gene expression.

In recent years, several experiments have shown that hammerhead ribozymes can induce a specific downregulation of the *MDR1* mRNA and P-glycoprotein resulting in reversal of the MDR-associated phenotype (Table 2). Kobayashi and colleagues [81, 82] reported the efficacy of two different anti-*MDR1* ribozymes driven by a pHβ promoter [83] in an acute leukaemia cell line, and in an *in vitro* cleavage condition. Their ribozymes have abrogated the cellular resistance to trimetrexate and vincristine. Scanlon and colleagues [84, 85] also demonstrated the efficacy of an anti-*MDR1* ribozyme driven by a pHβ promoter and an anti-*FOS* ribozyme driven by a dexamethasone-inducible promoter; both the anti-*MDR1* and anti-*FOS* ribozymes were transfected into a human ovarian carcinoma cell line resistant to actinomycin. The target site of their anti-*MDR1* ribozyme was chosen between the two ATP binding sites, which is suggested to be important for the function of the P-glycoprotein [86]. The anti-*FOS* ribozyme has suppressed *C-FOS* gene expression and also *MDR1* more efficiently than the anti-*MDR1* ribozyme. These data imply the potential significance of the *C-FOS* gene in the MDR phenotype. Holm and coworkers [87] transfected the same anti-*MDR1* ribozyme into a human pancreatic carcinoma cell line. The ribozyme completely suppressed *MDR1* gene expression and resulted in 300-fold reversal of daunorubicin resistance as compared to the parental resistant cell. Kiehntopf and colleagues [79] used a liposome-mediated transfer system for an anti-*MDR1* ribozyme to overcome the *MDR1*-associated multidrug resistance. The ribozyme reversed both the overexpression of the P-glycoprotein and the drug resistance level of a human pleural mesothelioma cell line.

Some groups have described the *in vitro* activity of anti-*MDR1* ribozymes. The first group designed fluoro- and allyl-substituted ribozymes in order to increase their stability against ribonucleolytic attack [61]. They also showed that these modified ribozymes could reduce the chemoresistance to doxorubicin in a human colorectal carcinoma cell line by up to 50%. The second group demonstrated the *in vitro* cleavage conditions of an anti-*MDR1* ribozyme [88]. Their catalytic activity was dependent on the ribozyme to target ratio, pH, $MgCl_2$ concentration and incubation time.

Based on recent findings, it is suggested that ribozyme-mediated specific antitumour therapy could be an effective strategy for reversing the MDR phenotype. However, there remain issues which have to be resolved [90]. The investigation and identification of an appropriate delivery system and targeting [91] may lead to the success of ribozyme-mediated therapy of the MDR phenotype.

DELIVERY SYSTEMS OF ODNs AND RIBOZYMES

The optimal delivery of ribozymes or ODNs to the tissue of interest is necessary in order for them to be effective. The issue of delivering these molecules to a particular cell selectively and efficiently has become an ongoing challenge in the clinical application of gene therapy for oncological disease processes [92]. Non-viral methods have been identified and studied extensively [93]. These involve various means of physical protection of the ribozymes or ODNs for safe delivery into the cell. Protection from the renal filtration system, systemic enzymatic degradation and immunological processes is necessary for the ribozymes or ODNs to reach the targeted cell and exert their ultimate effect. Particle bombardment has been used as a physical method to transfer DNA into a host cell by coating the DNA with mineral beads. The physical force of delivery overcomes the membrane barrier and allows entry into the cellular milieu. Although it has been found to be efficient both *in vitro* and *in vivo* [94], it theoretically would require an invasive surgical procedure for tissue exposure to allow the appropriate mechanical delivery. Also, direct injection of DNA or RNA has been shown to be an effective means

of gene transfer in susceptible tissues. However, muscle has been found to be the most efficient targeted tissue [95] and limits its potential use. It is used at present for the *in vivo* transfer of the Duchenne muscular dystrophy gene (DMD) [96].

Another means of gene transfer is the use of cationic lipids [97]. Cationic lipids form a particle in which the DNA or RNA is trapped through ionic attractions between the negatively charged DNA or RNA and the positive charges of the cationic lipids. Advantages of this method include its efficacy, lack of limitation of DNA or RNA size, lack of immunogenicity, easy preparation and absence of integration into the host's genome which obviates the possiblity of insertional mutagenesis. This method holds promise for future non-viral-based delivery systems. One disadvantage currently being studied is the toxicity of the liposome formulation in high concentrations. Modifications in the lipid carrier will hopefully reduce this drawback. Receptor-mediated transfection has also been investigated by associating ribozymes or ODNs with molecules that are capable of binding to the cell surface and facilitating endocytosis. Transferrin- and polylysine-DNA complexes have been found to exhibit good transfection efficiency *in vitro* in haematopoietic cells and other cell types [98–101]. However, early release of the ribozymes or ODNs from the endosomes is a potential concern. The use of adenovirus and haemagglutinin has been documented to improve the efficiency of transfer [102–107].

Viral agents use the cells inate biochemistry to express a specific ribozyme or ODN. For the ribozyme or ODN to be expressed, it is cloned into a plasmid or a viral vector and delivered by transfection or retroviral infection. Plasmids have been used as efficient means of ribozyme or ODN delivery. The plasmid, pHβ Apr-1-neo, driven by the human β-actin promoter [83] has been used for various ribozymes. For example, the anti-*FOS* ribozyme has been cloned into the pMAMneo vector containing the MMTV dexamethasone-inducible promoter [85]. This expresses transient amounts of the ribozyme when compared with the pHβ Apr-1-neo vector, but accomplishes efficient downregulation of the targeted *FOS* gene. Retroviral vectors associated with the murine leukaemia virus and family are the oldest and the most clinically experienced viral system thus far [108]. They offer stable integration into the host cells. However, high titres of virus are needed for optimal transfection efficacy. The activity of retroviral vectors is limited to actively dividing cells. Adeno-associated virus-based vectors have also been studied for gene-mediated transfer into cells [109]. Adeno-associated virus is a non-autonomous parvovirus not known to be involved in any pathological condition. It provides stable integration and long-term infection in transfected cells. It may be expressed in both dividing and non-dividing cells. The adeno-associated virus normally requires a helper virus for expression, usually the adenovirus or the herpes simplex virus (HSV) and may infect a wide variety of cells including cells of haematopoietic lineage. Another experimental vector has been developed from the herpes simplex-1 (HSV-1) genome [110]. HSV-1 belongs to the E-type subfamily of herpes virus. It offers many advantages including, wide host availability, high titre stocks, and a large insert capacity. HSV-1 does not integrate into the host genome, but persists in some cells in a non-integrated latent state. Toxicity is the limiting factor for this type of vector and is due to the expressed HSV proteins. Many modifications of the HSV vectors have been unsuccessful at minimising this disadvantage and thus HSV has limited clinical application at present.

Adenovirus vectors are currently being used in several clinical trials and hold promise as efficient delivery systems in gene therapy [111]. The adenovirus consists of a family of viruses (more than 40 serotypes) that are responsible for minor respiratory infections in humans. Several advantages of this vector include high titre levels, a benign clinical profile (based on its use in the past as a vaccine against adenovirus respiratory infection by the U.S. Army), and its high levels of transgene expression. Adenovirus may be expressed in a broad range of cell types and efficient transduction may be observed in both replicating and non-replicating cells.

Many delivery systems exist, as demonstrated above. The challenge in gene therapy today, lies in the identification of minimal toxicity, high efficiency for the specific delivery of ribozymes or antisense ODNs on the targeted cell of interest.

CONCLUSION

The recent development of antisense and ribozyme technology has made it possible to use these molecules as potent modulators of the MDR phenotype. Many conclusive results have demonstrated the potential of antisense ODNs and ribozymes to modulate the MDR phenotype in many types of resistant cancer cells. The crucial event that will bring these molecules to the forefront of cancer therapy will be the development of proper delivery systems and targeting. Antisense ODNs and ribozymes can now be seen as important new therapeutic drugs and it can be anticipated that these molecules will emerge as one of the most effective strategies for modulating the MDR phenotype in human cancer in the near future.

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