Recent Attempts at RNAi-Mediated P-Glycoprotein Downregulation for Reversal of Multidrug Resistance in Cancer

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Published online 26 April 2010 in Wiley Online Library (wileyonlinelibrary.com).
DOI 10.1002/med.20244

Abstract: Multidrug resistance (MDR) is among the major mechanisms leading to failure in chemotherapy of cancer patients. The ATP-binding cassette proteins are major contributors to MDR, involved in the active efflux of xenobiotics out of cancer cells. Among them, P-glycoprotein (P-gp) is the most dominant protein involved in the efflux of drugs. For more than 30 years, scientists have searched for the ideal P-gp inhibitor to modulate drug resistance activity of P-gp. This inhibitor should be tissue and cell specific with side effects on other tissues, must not provoke immune responses from the host, should provide sustained inhibition, and must be synthesized readily with low cost. Chemical P-gp inhibitors tested to date, have shown nonspecific toxic effects limiting their clinical applications. Sequence-specific P-gp gene silencing by RNA interference (RNAi) may provide a more effective approach for downregulation of specific protein targets due to high specificity, limited toxicity and immunogenicity, and relative ease in synthesis. RNAi can be implemented by delivery of synthetic small interfering RNAs (siRNAs) or by gene expression of short hairpin RNAs using gene expressing vectors. Specific delivery systems and expression vectors have been designed for this purpose and many researchers have explored their effectiveness for P-gp downregulation. In this report, we review the efficiency of various methods for siRNA delivery and transfection for P-gp downregulation in cancer cells for MDR reversal. Novel ideas and observations by different research groups were discussed for future improvement in this essential field. © 2011 Wiley Periodicals, Inc. Med Res Rev., 33, No. 1, 33–53, 2013

Key words: siRNA; multidrug resistance; P-glycoprotein; cancer; chemotherapy

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Contract grant sponsors: Canadian Institutes of Health Research (CIHR); Natural Sciences and Engineering Council of Canada (NSERC); Alberta Advanced Education and Technology (AET); Alberta Heritage Foundation for Medical Research (AHFMR).

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1. DRUG RESISTANCE AND P-GLYCOPROTEIN

Resistance to drugs is among the major causes of failure of cancer chemotherapy. Major mechanisms of drug resistance include (1) decreased intracellular uptake of soluble drugs, (2) genetic and phenotypic changes in cells that change the capacity of drugs to cause the desired cell damage, and (3) increased efflux of especially hydrophobic drugs by cell-surface transporters, leading to multidrug resistance (MDR).[1] As most chemotherapeutic drugs are hydrophobic, MDR is consistently detected in tissues undergoing chemotherapy and it causes simultaneous resistance to different drugs with different targets and chemical structures.[2] This phenomenon can be either intrinsic or acquired as a result of exposure to chemotherapeutic agents. Acquired MDR is a common occurrence in breast and ovarian cancers where 50–70% patients typically display MDR.[3] One reason for acquired resistance is the insufficient drug concentration exposed to tumor cells, so that the cells adapt to low drug concentrations without undergoing drug-induced cell death. A relatively short circulation time of chemotherapeutic agents limiting their access to tumor mass, as well as limited drug diffusion into the interstitial spaces of tumors, could lead to such a low drug concentration in situ. Phenotypic alterations in the tumor cells could also cause the acquired MDR, as tumor cells change their membrane surface composition at different stages of neoplastic growth.[4]

The main efflux pumps involved in MDR are ATP-binding cassette family members, including P-glycoprotein (P-gp), multidrug resistance protein 1, and breast cancer resistance protein. The P-gp is the most common protein involved in ATP-dependent efflux of drugs in various cancerous tissues.[2,5] P-gp is normally present at the apical surface of epithelium lining the colon, small intestine, bile ductules, and kidney proximal tubules, where it secretes xenobiotics and various metabolites into bile, urine, and lumen of gastrointestinal tracks. P-gp is present on endothelial cells of the blood–brain barrier, blood–testis barrier, and the blood–ear barrier, where it protects these susceptible organs from toxic xenobiotics. P-gp consists of two membrane-bound domains, each made of six transmembrane helices and two cytoplasmic nucleotide-binding sites that bind and hydrolyze ATP. In 2009, X-ray structure of mouse P-gp was reported in the drug-binding-component state.[6] In this model, the P-gp structure is composed of two halves with molecular symmetry, measuring ~136 Å perpendicular to and ~70 Å in the plane of the membrane. The intracellular conformation is composed of two bundles of six transmembrane helices, forming a large cavity open to the cytoplasm and the inner membrane in accordance with the vacuum cleaner hypothesis. Additionally, the internal domain contains the nucleotide-binding domain (ATP binding sites) and two portals for the entry of hydrophobic molecules. The internal cavity within the lipid bilayer is significantly large and takes up compounds simultaneously.[7] This cavity can become even larger to accommodate larger substrates. The P-gp has a broad substrate family ranging from drugs to biological compounds.[8] Several classes of chemotherapy drugs, including vinca alkaloids, anthracyclines, epipodophyllo toxin, and taxanes, are effective substrates of P-gp. The substrate-binding pocket of P-gp consists of hydrophobic and aromatic residues. Substrates and specifically drugs are extracted from the cytoplasm and lipid bilayer,[9] and lipids are needed for ATPase activity required for drug efflux.[10] ATP binding is stimulated by substrate binding to the its binding site on P-gp. This likely causes a dimerization in the nucleotide-binding domain (NBD), which produces large structural changes resulting in an outward-facing conformation. Release of substrate is either due to decreased binding affinity or facilitated by ATP hydrolysis. In either case, ATP hydrolysis disrupts NBD dimerization, resets the system back to inward facing conformation, and reinitiates the transport cycle.[11]

The P-gp has been successfully subdued with chemical inhibitors in the last three decades. Based on their affinity and specificity for P-gp, the chemical inhibitors have been divided into three generations.[12] First-generation inhibitors consist of calcium channel blockers (e.g., verapamil) and immunosuppressants (e.g., cyclosporine A). Their drawback is that they are highly

toxic at effective doses for P-gp inhibition due to off-target activities. The second-generation inhibitors have higher specificity and affinity to P-gp, and thus are more effective than the first generation. They consist of analogs of verapamil, dexniguldipine, and valspodar (PSC 833). Although they show limited off-target activity, they are substrates for cytochrome p450 and interfere with the function of this enzyme, which is required for the metabolism of xenobiotics and especially drugs. This causes a dramatic increase in toxicity by the coadministered drugs. The third generation P-gp inhibitors show enhanced selectivity, limited toxicity, and minimal pharmacokinetic interactions. These include the anthranilamide derivative tariquidar (XR9576), elacridar, diketopiperazine derivative XR9051, cyclopropyldibenzo-suberane zosuquidar (LY335979), substituted diarylimidazole ONT-093, and biricodar (VX-710). A drawback associated with a high dose of this group of inhibitors is the nonspecific inhibition of Breast Cancer Resistance Protein (ABCG2). This may result in toxicity as the ABCG2 transporter has protective roles the intestine, blood–testis barrier, blood–brain barrier, and the membranes of hematopoietic progenitor and other stem cells. Several reviews have focused on clinical significance of P-gp inhibition and various P-gp modulators,[12–14] so that the reader is referred to this literature for more in-depth discussion on this topic.

Increased ubiquitination of P-gp has been shown to cause P-gp degradation, leading to reduced MDR and increased drug accumulation inside cells. Zhang et al. have used ubiquitin transfection of MDR1 positive cells to modulate the ubiquitin–proteasome pathway and reverse MDR in cancer cells.[15] Additionally, phosphorylation of P-gp by protein kinase C has been reported to increase efficiency of P-gp-mediated drug resistance,[16] indicating that protein kinase C may be involved in development P-gp-mediated drug resistance. Furthermore, p53 tumor suppressor gene has been proposed to reverse MDR by reducing P-gp phosphorylation through the transcriptional repression of PKC expression.[17] These findings highlight the significance of P-gp ubiquitination and the PKC-mediated modulation of P-gp phosphorylation as new targets for the reversal of MDR.

2. SPECIFIC DOWNREGULATION OF P-gp BY RNA INTERFERENCE

Unlike chemical regulators, RNA interference (RNAi) technology may provide a more specific approach the downregulation of protein targets, such as P-gp. The clinical potential of RNAi is actively explored in cancer therapy based on synthetic analogs of RNAi, namely short interfering RNAs (siRNAs). The siRNAs are usually double-stranded, 21–25 nucleotide-long molecules, generated through the breakdown of long dsRNAs by enzymes known as dicers. siRNA is then activated by binding to RNA induced silencing complex (RISC), which unwinds the siRNA duplex and produces an oligonucleotide that binds to a specific sequence on the target mRNA, leading to cleavage and disposal of the resulting dsRNA.[18] Among the potential advantages of using siRNA are its reduced toxicity on nontarget tissues, as compared to the conventional P-gp inhibitors and high degree of specificity on desired gene targets.[19,20] The G/C content of siRNA has been reported to be significant in siRNA efficiency for P-gp downregulation. It has been reported that efficient siRNA sequences contain 30–52% G/C content. The presence of A/U at the 5′ end of the antisense strand was suggested to increase efficiency,[21,22] where 3–5 A/Us at the antisense strand were beneficial. Low internal stability of the siRNA at the 5′-end of the antisense strand was also considered important for duplex unwinding and efficient entry into RISC sequences.[23]

Other molecules that may be useful in specific downregulation of P-gp are micro-RNAs (miRNAs),[24] hammerhead ribozymes,[25] and antisense oligonucleotides (ASO)[26] (Table I). miRNAs are single-stranded 19–25 nucleotides produced from hairpin RNAs by dicer activity. They are partially complimentary to mRNA and inhibit translation by binding to various mRNA sequences. As in siRNA, miRNA function is associated with the RISC

Table I. Summary of Published Reports That Used Delivery of Molecules Other Than siRNA for Sequence-Specific Downregulation of P-gp

Reference	Cell line	Therapeutic molecule	Carrier	Dose	P-gp suppression	Tumor suppression/drug
Pakunlu et al.[26]	MCF7 Cells	Antisense oligonucleotide	Liposome	500 μM	80% P-gp suppression	P-gp expression in MCF-7 cells was decreased by three-fold
Nagata et al.[25]	Human colon cancer cell line (HCT-8DDP)	Hammerhead ribozymes	pHβ plasmid	1.4 μM	50% P-gp mRNA suppression	2.5 and 4.1-fold increased sensitivity to doxorubucin and etoposide. Resistances to cisplatin, methotrexate, and 5-fluorouracil were not affected
Motomura et al.[29]	Myelogenous adriamycin-resistant leukemia cells K562/ADM[16]	Cationic liposome (Lypolymine)	Lipofectamine™ 2000	2.5 to 20 μM	25–75% P-gp downregulation	Chemosensitivity to daunorubicin increased up to 5.9-fold
Zhu et al.[24]	Human ovarian cancer multidrug resistant A2780DX5	miRNA	Oligofectamine™	NA	Suppressed P-gp levels by 40%	Increased sensitivity to vinblastine by ~50%

multiprotein complex.[27] Using miRNA for P-gp downregulation and Oligofectamine™ for delivery, Zhu et al. were able to suppress P-gp levels by 40% in the human ovarian cancer cell line, A2780, and its multidrug resistant counterpart, A2780DX5. They further showed that P-gp downregulation by the chosen miRNA increased sensitivity to vinblastine by ~50%.[24]

Hammerhead ribozymes are 30–40 nucleotide long, catalytic RNAs that are able to control gene expression by eliminating specific RNAs. They are functional after expressing ribozymes within target cells or by delivering the ribozymes to cells as a preformed entity.[28] The structure of hammerhead ribozymes contains three base-paired stems and a highly conserved core of residues required for RNA cleavage. Using hammerhead ribozymes against MDR1 mRNA in a human colon cancer cell line (HCT-8DDP), it was possible to obtain 2.5 and 4.1-fold increased sensitivity to doxorubicin (DOX) and etoposide (VP-16), respectively. Resistance to cisplatin, methotrexate, and 5-fluorouracil was not affected in these cases.[25]

The ASOs are nucleotide sequences complementary to a target mRNA, which recruit RNase to cleave the target mRNA, ultimately blocking mRNA translation. ASOs were employed for simultaneous downregulation of MDR-1 and Bcl-2 for increased accumulation of DOX in drug-resistant MCF-7 cells. With a liposomal system for simultaneous delivery of ASOs and DOX, P-gp expression in MCF-7 cells was decreased threefold.[26] A high concentration (500 μM) of ASOs was needed for effective downregulation, which was reported to have off-target toxicities. Motomura et al. used a cationic liposome (Lypolymine) to deliver ASOs to K562 myelogenous leukemia cells and its adriamycin-resistant phenotype K562/ADM;[16] 25–75% P-gp downregulation was achieved at 2.5–20 μM ASO concentrations.[29]

3. EFFORTS ON siRNA-MEDIATED DOWNREGULATION OF P-gp

A. Critical Considerations for siRNA Delivery

For an efficient RNAi and protein knockdown, several successive steps have to be successfully completed. Initially, extracellular siRNA has to cross the lipid bilayer comprising the plasma membrane. A carrier with high affinity to siRNA is required for this purpose, as siRNA cannot cross cellular membranes on its own due to its anionic nature. This carrier is expected to protect siRNA against extra- and intracellular nucleases. It is believed that most siRNA–carrier complexes adsorb and enter the cell through nonreceptor-mediated endocytosis.[30] The complex will most likely be transported in endosomes, where siRNA has to be protected from degradation by the endosomal/lysosomal system and somehow escape this entrapment. After the endosomal escape, siRNA has to dissociate from its carrier (if still present as a complex) for interactions with the RISC multiprotein complex. The optimal outcome of this event is the specific binding of siRNA to target mRNA, and ultimately target mRNA cleavage and degradation. In the case of the MDR1 gene, successful siRNA delivery is expected to lead to decreased MDR1 expression, reduced population of P-gp molecules on cell surface, reduced efflux of chemotherapeutic drugs from cells, and increased concentration of drugs in treated cells, resulting in increased cellular necrosis and/or apoptosis (Image 1).

Another barrier to effective downregulation of P-gp is the long half-life (14–17 hr) of P-gp protein.[13,14] Downregulation of P-gp by siRNA limits the production of new P-gp, but a therapeutic effect is observed only when the overall P-gp population is reduced (exceptions were noted[31]). Naked siRNA is rapidly degraded in physiological milieu and has a very short half-life (hours). Thus, siRNA persistence in cells is critical for P-gp silencing. The combined effect of short duration of downregulation from the synthetic siRNAs and long target half-lives is partial reduction of P-gp activity. Stable expression of MDR1 shRNAs targeting different regions of the coding sequence has been shown to be more effective against long-lived proteins.[32] In order to

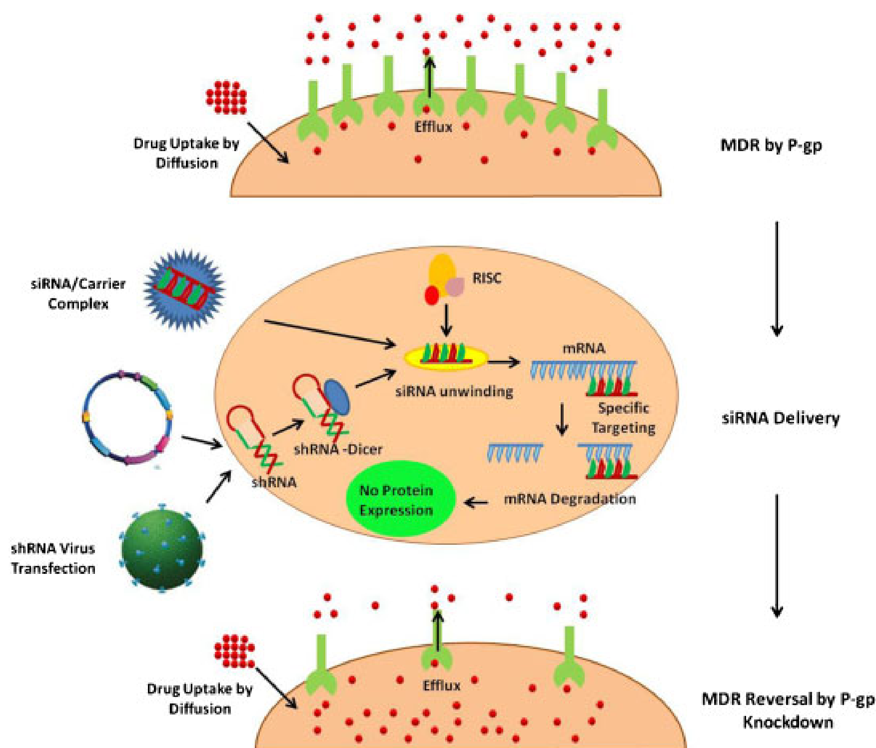

Image 1. **A.** MDR by P-gp is depicted showing efflux of chemotherapeutic drugs out of the tumor cells. **B.** Mechanism of siRNA delivery and function is shown leading to suppressed P-gp expression. **C.** MDR reversal by siRNA mediated P-gp down-regulation is shown, leading to increased concentrations of chemotherapeutic drugs inside the tumor cell.

increase the half-life and bioavailability of synthetic siRNAs, various groups have made efforts to chemically modify siRNA. The interactions of siRNA with RISC complex were shown to be relatively insensitive to the 2′-OH end of molecule, so that 2′-fluoro and 2′-OMe modification of siRNA was attempted to increase siRNA half-life in cytoplasmic space, without limiting its silencing ability.[33] Furthermore, by strengthening the U-A linkage and modifying the P-S backbone linkages, this group has shown that siRNA stability in serum can be increased with no effect on silencing activity.[34] Layzer et al. compared the half-life of 2′-Fluoro-modified siRNA to unmodified 2′-OH-siRNA and evaluated the kinetics of gene silencing in HeLa cells. Their modification increased siRNA half-life in serum from 24 to >72 hr; however, this modification did not affect gene silencing after siRNA treatment.[35] Rapidly dividing HeLa cells might have diluted the siRNA excessively in this study. These modification methods might be especially significant when siRNA silencing is performed in vivo, as RNases are at high concentrations in the lymph, blood, and extracellular matrix. Additionally, a drawback that has been associated with the in vivo siRNA knockdown of target genes is the activation of the innate immune system by the activation of toll-like receptors (TLR) due to siRNA recognition in the endosomes. This process, which is used in mammalian cells as a defense mechanism against viral RNA,[36] is sequence specific and has rarely been reported in in vitro delivery of siRNA. However, several groups have reported this phenomenon in the in vivo silencing studies, which may misrepresent therapeutic effects of siRNA. Activation of the immune system by TLR3 has been reported by Kleinman et al., and they have attributed the therapeutic effect of siRNA on choroidal neo-vascularization to the immune response arisen by the activation of this receptor.[37] Other groups have suggested that TLR7 and TLR8 receptors recognize siRNA, and activate cytokines and

interferons of the innate immune system that may lead to therapeutic effects.[38–40] These factors are important considerations that complicate therapeutic actions of siRNA.

Even though siRNA action is sequence specific, siRNA-mediated silencing was often associated with off-target gene activation. The reason behind this phenomenon is not exactly clear; however, similarities in mRNA sequences between the target sequence and other unrelated sequences might give rise to this undesired response.[41] dsRNA can also activate several protein kinases, such as p38, JNK2, IKK, and PKR.[42] Induction of these signaling pathways can alter gene expression in an unpredictable way by regulating the activity of transcription factors, such as NF-κB, IRF-3, and ATF-1.[43] This off-target activity can be reduced by employing low siRNA concentrations for silencing. Persengiev et al. reported an increase as well as a decrease in the expression of various mammalian genes in response to a luciferase siRNA treatment (where no natural target is expected to exist). They observed a concentration-dependent effect of siRNA in various genes with siRNA concentrations at >25 nM.[44] Semizarov et al. also observed off-target effects of siRNA at 100 nM, but not at the 20 nM.[45] Therefore, the reported data strongly favor the use of <20 nM siRNA concentrations for MDR1 downregulation to minimize off-target effects.

Most siRNA concentrations reported for MDR reversal have been >100 nM (Fig. 1). This figure summarizes the reported efficacy studies based on the siRNA concentration that yielded maximal P-gp downregulation. It can be seen that the lower siRNA concentrations were as effective as the higher concentrations for P-gp downregulation. It should be noted that there were significant variations in the phenotype of target cells and delivery methods in these studies, and this is explored in the next section.

B. Carriers for Efficient siRNA Delivery In Vitro

A carrier is a necessity for effective siRNA delivery and several types of carriers have been explored for this purpose. Lipid-based carriers have been among the most commonly used carriers for siRNA delivery in P-gp downregulation (Table II). The ease of synthesis and

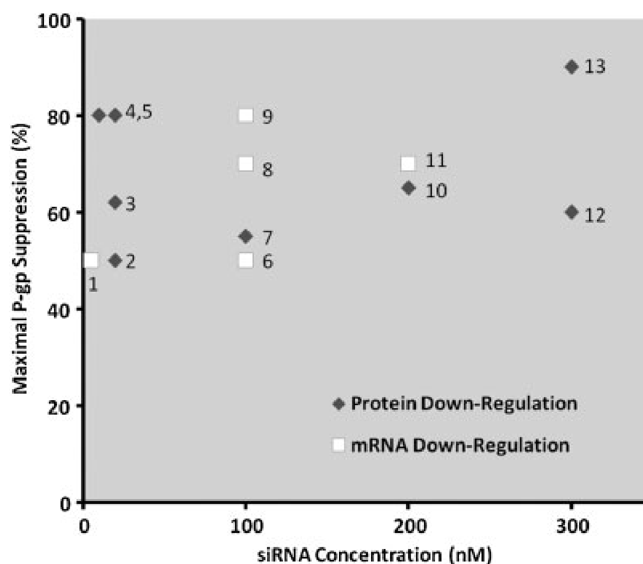

Figure 1. The siRNA concentrations used for maximal downregulation of the P-gp at the mRNA and protein levels in various cell lines. Results from representative (not exhaustive) studies were provided. A similar siRNA concentration was generally effective for P-gp downregulation at both mRNA and protein levels. In addition, a relatively lower concentration of siRNA was as effective as the high concentrations for P-gp downregulation. The numbers in the figure correspond to the following references: 1 = 36, 2 = 39, 3 = 35, 4 = 34, 5 = 36, 6 = 52, 7 = 47, 8 = 47, 9 = 50, 10 = 38, 11 = 56, 12 = 46, 13 = 59.

Table II. Summary of Published Literature That Employed siRNA Delivery for In Vitro P-gp Suppression

Reference	Cell line	Carrier	siRNA	siRNA dose	P-gp suppression
Xiao et al.[62]	Human lung carcinoma cell line NCI-H460	Lipofectamine™ 2000	StealthTM RNAi 3 (5′-AACUUGAGCAGCAUCAUUGGCGAGC-3′)	100 nM	50–79% P-gp mRNA suppression
Xing and Wang[68]	Human ovarian cancer cell lines, A2780, CAOV3, SKOV3, and SW626	Oligofectamine™/ Lipofectamine™ 2000 complex	5′GATCCCATTCGCTATGGCCGTGAATTCAAG AGATTCACGGCCATAGCGAATGTTTTTG-3′ & 5′-GCTTTTCCAAAAAACATTCGCTATGGCCG TGA ATCTCTTGAATTCACGGCCATAGCGAC TTAA-3′	200 nM	70–80% P-gp mRNA suppression
Stierle et al.[47]	Doxorubicin-resistant cell line MCF7-R cells	Oligofectamine™	S1: 5′-GAAACCAACUGUCAGUGUA	20 nM	~55% P-gp suppression at 48 hr, 45% at 72 hr
			S2: 5′-CUUUGGCUGCCAUCAUCCA		~35% P-gp suppression at 48 hr, no effect at 72 hr
			S1+S2		~60% P-gp suppression at 48 hr, no effect at 72 hr
Li et al.[46]	MCF7/R cells	Lipofectamine™ 2000	Endonuclease-prepared small interfering RNA (EsiRNA)	10 nM	~80% P-gp suppression
Stierle et al.[48]	MCF7-R	Oligofectamine™	Si_1 5′-UACACUGACAGUUGGUUUCd TdTdTdTAUGUGACUGUCAACC AAAG-5′ Si_2 5′-UGGAUGAUGGCAGCCAAAG dTdTdTdTACCUACUACCGUCGGUUC-5′ Si_3 5′-UACGGUGCAAUUCGUAUCdTdT dTdTdTAUGCCACAGUUAAGCAUAG	5 nM	Maximum of 50% P-gp mRNA suppression
Zhang et al.[67]	MDR human ovarian cancer cells, COC1/DDP	Oligofectamine™	MDR-1: 5V-AAGGAAAAGAAACC AACTGTC-3V;	150 nM	Exact extent not given

Wu et al.[50]	MCF-7/AdrR and MCF-7/BC-19	Oligofectamine™	5′-GGAAAAGAAACCAACUGUCdTdT(sense) dTdTCCUUUUCUUUGGUUGACAG-5′(antisense)	200 nM	Maximum of 65% P-gp mRNA suppression
Stierle et al.[48]	NIH-3T3 cells	Oligofectamine™	(Antisense, 5′-UACACUGACAGUUGGUUUCdTdT; sense, 5′-GAAACCAACUGUCAGUGUAdTdT)	20 nM	Maximum of 80% P-gp suppression in MCF7 cell
	NIH-MDRG185 Cells MCF7-S		(Antisense, 59-UGGAUGAUGGCAGCCAAAGdTdT; sense, 59-CUUUGGCUGCCAUCAUCCAdTdT).		
	MCF7-R		(Antisense 59UACGGUGUCAAUUCGUAUCdTdT; sense, 59 GAUACGAAUUGACACCGU-AdTdT)		
Huaa et al.[71]	Multiple drug-resistant cell line MES-SA/DX5 (ATCC CRL-1977	Lipofectamine™ 2000	Sense: 5VAGGCCAACATACATGCCTTC 3V, anti-sense: 5VGCTCCTTGACTCTGCCATTC 3V, Sense: 5VAACTGGGACGACATGGAQGAA 3V, anti-sense: 5VAGAGGCGTACAGGGATAGCA 3V	300 nM	~90% P-gp suppression after 2 month transfection
Abbasi et al.[51]	MDA-435/LCC6 melanoma cell line	Stearic acid-poly(L-Lysine) (PLL-StA)	Sense: 5′-CAGAAAGCUUAGUACCAAAdTdT, antisense: UUUGGUACUAAGCUUUCUGTC-3′.	20 nM	~50% P-gp suppresion
Xiong et al.[58]	MDA-435/LCC6 melanoma cell line	PEO-*b*-P(CL-*g*-TP) PEO-*b*-P(CL-*g*-SP)	Ambion ABCB1 siRNA	100–300 nM	50–60% p-gp down-reg
Xiong et al.[59]	MDA-435/LCC6 melanoma cell line	RGD4C-PEO-b-P(CLg-DP), TAT-PEO-b-P(CL-g-DP)	Ambion ABCB1 siRNA	100 nM	70% P-gp mRNA Suppression, 55% P-gp suppression by flow
Patil et al.[64]	JC drug-resistant tumor cell line	Poly(D,L-lactide-co-glycolide) nanoparticles	Sense: 5′-GAGTGAGGCCGATAAAAGAGC CATGTT Antisense: TCATCTGTGAGCCGGGTGTTGA GCTCCC-3	100 nM	50% P-gp mRNA suppression
Patutina et al.[63]	MDR positive RLS40 cell line	Lipofectamine™ 2000	Sense 5′-GGCUGGACAAGCUGUGCAUGG-3′, antisense 5′AUGCACAGCUUGUCCAGCCAA-3′	20–200 nM	P-gp mRNA suppression, exact extent not given

versatility makes them an appropriate choice for siRNA delivery and they have shown high efficiency in in vitro studies. However, several drawbacks are associated with these carriers, such as toxicity, immunogenicity, and lack of stability under physiological conditions. Lipofectamine™ 2000[46] and Oligofectamine™[47–50] were the most common lipid-based carriers and many groups have reported success in P-gp downregulation with these carriers in vitro (Table II). The proprietary formulation Oligofectamine™ has been used in three series of studies. First study delivered P-gp siRNA to yield 60–80% downregulation in MCF7 cells by using a combination of two siRNA sequences.[47,48] The latter was pursued with the expectation of better efficacy, but the P-gp downregulation observed with the combined siRNAs did not surpass the P-gp downregulation observed with single siRNAs. This study used a relatively low concentration of siRNA (20 nM); however, the consequences of P-gp downregulation on intracellular drug accumulation and cytotoxicity were not reported. A second study pursued P-gp downregulation in human gastric EPG85-257RDB and pancreatic EPP85-181RDB carcinoma cells (MDR established by in vitro exposure to daunorubicin). As much as 58 and 89% downregulation in protein levels were obtained in EPG85-257RDB and EPP85-181RDB cells, respectively. Intracellular drug accumulation and toxicity assays were again not reported, so that functional consequences of P-gp downregulation were unknown.[49] In a third study, P-gp siRNA delivery was pursued to MDR breast cancer cell lines, MCF-7/AdrR and MCF-7/BC-19, and their parental drug-sensitive MCF-7 cells. A maximum of ~65% P-gp downregulation was achieved at high (200 nM) siRNA concentrations, and ~two-fold increase in the accumulation of paclitaxel and DOX was achieved. Several reasons were suggested for not obtaining 100% P-gp inhibition: (1) high content of P-gp requiring excessive siRNA dose, (2) long half-life of P-gp protein, requiring prolonged presence of siRNA in cells, and (3) low delivery efficiency.[50] P-gp levels will inevitably vary in different cell lines (and more importantly in clinical samples), whose assessment may guide siRNA dose required for effective delivery. The duration of siRNA inhibition obtained by this group was ~24 hr and P-gp silencing was lost after 72 hr, consistent with the results obtained by the author's group.[51]

Cationic polymers have been pursued as alternatives to lipid carriers. Polyethyleneimine (PEI)-derived polymers served as the initial choice, given their strong interactions with nucleic acids.[52] The PEI provides stronger siRNA protection compared to lipid-based carriers and increases siRNA half-life in cytoplasm.[53,54] The high molecular weight PEI, however, displays significant cytotoxicity due to strong perturbations of cellular membrane that induce necrotic changes and ultimately lead to apoptotic changes.[55] Several modifications of PEI have been pursued to improve its delivery efficiency,[52,56] including lipid functionalization.[57] These modified PEIs were not evaluated for P-gp downregulation, but a lipid (stearic acid) functionalized poly(l-lysine) (PLL-StA) was shown to enhance siRNA delivery into drug-resistant MDA435/LCC6 cells, as compared to unmodified PLL (~ten-fold increase).[51] Furthermore, PLL-StA protected siRNA better than the purely cationic PLL against serum degradation and ultimately increased the siRNA half-life in cells. P-gp expression was reduced by 50–60% using this carrier when the cells were treated with a low concentration of siRNA (20 nM). This led to a three-fold increase in DOX accumulation in these cells and a ~30% increase in cytotoxicity. Lipofectamine™ 2000 showed variable results in our hands for P-gp downregulation (suppression was obtained at times but not consistently) and did not lead to a significant increase in drug uptake.[51]

A novel family of biodegradable polymers, namely poly(ethyleneoxide)-block-poly (3-caprolactone) (PEO-b-PCL) substituted with cationic side chains tetraethylenepentamine, spermine, and N,N-dimethyldipropylenetriamine, were employed for a micellar siRNA delivery system.[58] These polymeric micelles could be useful for systemic delivery to tumors, as their parent polymer (i.e., PEO-b-PCL) was designed for systemic administration. The

polymers were able to effectively bind to siRNA, protect it from nuclease degradation, and release the complexed siRNA efficiently upon interaction with anionic macromolecules. The siRNA formulated in cationic PEO-b-PCL micelles showed efficient cellular uptake into the drug-resistant MDA435/LCC6 cells and exhibited up to ~60% P-gp downregulation at 100–300 nM siRNA.[58] These are relatively high siRNA concentrations and may reflect the fact that this delivery system was micellar in nature and was designed to remain soluble in physiological fluids. To improve this delivery vehicle further, cationic PEO-b-PCL micelles were decorated with RGD4C (an integrin $\alpha_v\beta_3$-binding peptide) and TAT (a cell-penetrating peptide) peptides.[59] The RGD motif in the penton base of adenovirus is used for cellular internalization of viruses through binding to the $\alpha_v\beta_3$ integrins. HIV-1 TAT peptide is also used for binding of viruses to cells via interaction with cellular glycosaminoglycans. As some transformed cells overexpress $\alpha_v\beta_3$ integrin,[60] this approach could be useful for targeting cancer cells for a more effective gene silencing, ultimately lowering the siRNA concentration needed. Compared to nondecorated micelles, RGD- and TAT-decorated micelles resulted in ~two-fold increase in the percentage of siRNA-positive cells and ~70% downregulation in P-gp mRNA and ~55% reduction in P-gp protein levels at 100 nM siRNA. Drug accumulation was accordingly increased with peptide-decorated micelles; ~two-fold increase in DOX uptake was observed, leading to a three-fold increase in cytotoxicity.[59]

Another polymeric carrier designed for systemic siRNA administration was developed from a combination of a small (1.2 kDa) PEI, polyethyleneglycol (PEG) and hydrophobic (oleoyl; C18) lipid segments designed to link the PEI and PEG moieties.[61] This carrier successfully delivered P-gp siRNA to drug-resistant colon carcinoma CD133+ cells. Minimal toxicity, enhanced siRNA stability, and improved delivery were seen with this carrier, especially at optimal PEI:lipid ratios. A ~70% P-gp mRNA knockdown was shown as well as a reduction in P-gp protein levels (not quantitated) at 2.25 μg/ml siRNA (~200 nM). The relatively high concentration employed for effective P-gp silencing was consistent with PEO incorporating micellar systems designed for systemic administration. Paclitaxel treatment after siRNA delivery showed a ~two-fold reduced IC_{50} on target cells.[61]

C. siRNA-Mediated P-gp Downregulation in Animal Models

While many groups have successfully reversed MDR by P-gp downregulation in vitro, a limited number of studies pursued nonviral siRNA delivery in animal models (Table III). One group employed Stealth™ RNAi delivery system for P-gp downregulation in human lung carcinoma NCI-H460 cells, which is proposed to display less off-target activities due to chemical inactivation of sense strand. While Lipofectamine™ 2000 was used in feasibility studies in vitro, which yielded ~60% P-gp downregulation at 100 nM RNAi, delivery into tumors in nude mice was accomplished by electroporation without a carrier. A maximal P-gp downregulation of ~80% was seen in tumors, which resulted in a maximal reduction of ~60% in tumor size after 13 days of vinorelbine treatment.[62] It should be noted that a high degree of variation was seen in the size of drug treated, no siRNA receiving tumors in this study (0.4–1 cm^3). Lipofectamine™ 2000, however, was successfully employed in another study, where intraperitoneal injection of siRNA/lipid complexes resulted in ~80% downregulation of P-gp levels in a murine model.[63] Another nonviral delivery approach relied on biodegradable poly(D,L-lactide-co-glycolide) nanoparticles for simultaneous delivery of P-gp siRNA and chemotherapeutic drug paclitaxel.[64] Being hydrolytically degradable, the nanoparticulate system provided sustained release of both the siRNA and paclitaxel over a period of 2–4 weeks. While in vitro studies yielded ~50% reduction in P-gp, a single systemic injection of biotin-decorated nanoparticles (with 20 μg P-gp siRNA and biotin for tumor targeting) was able to reduce the growth of JC breast cancer tumors in BALB/c mice by 50%

Table III. Summary of Published Literature That Employed siRNA Delivery for In Vivo P-gp Downregulation and Tumor Suppression

Reference	Animal/tumor	Carrier	siRNA	siRNA dose	P-gp suppression	Tumor suppression/drug
Xiao et al.[62]	Balb/c nude mice bearing NCI-H460 xenografts	Electroporation	Stealth™ RNAi 3(5′AACUUGAGCAGCAUCAUUGGCGAGC-3′)	20, 40, 80, 120 μM	80% P-gp suppression	60% Tumor suppresion after 13 days of navelbine treatment
Patil et al.[64]	Balb/c nude mice bearing JC xenografts	Poly(D,L-lactide-co-glycolide) nanoparticles	Sense: 5′GAGTGAGGCCGATAAAAGAGCCATGTT Antisense: TCATCTGTGAGCCGGGTGTTGAGCTCCC-3	1.4 μM	50% P-gp mRNA suppression	50 Tumor suppresion after 16 days of Paclitaxel treatment
Patutina et al.[63]	Lymphosarcomas in CBA/LacSto mice	Lipofectamine™ 2000	Sense 5′-GGCUGGACAAGCUGUGCAUGG-3′, Antisense 5′AUGCACAGCUUGUCCAGCCAA-3′	850 nM	4 fold decrease in P-gp mRNA	Embichin → 1.4-fold smaller tumors at 15 days Cyclophosphamide → 3.3-fold smaller tumors after 15 days

after 16 days.[64] The tumors were not responsive to paclitaxel and the desired response was seen only after P-gp downregulation by the nanoparticles. The efficacy obtained in this study was reminiscent of the outcome with the intratumor application of P-gp Stealth™ RNAi,[62] but the feasibility of achieving the desired therapeutic effect after a single systemic injection is a significant benefit.

4. EXPRESSION OF P-gp SILENCERS IN SITU

In situ expression of silencer molecules has been pursued for longer term RNAi in comparison to short-lived siRNA action. This approach employs vectors for small hairpin RNA expression (shRNA, which is ultimately processed into siRNA) or expression cassettes producing sense and antisense strands that hybridize to form functional siRNA in target cells.[65] As with siRNA, functional carriers are needed to deliver the expression vectors into target cells. ShRNA against P-gp was expressed in KD30 leukemia cells by using the pSUPER expression vector, which resulted in $>90\%$ suppression of P-gp mRNA and protein levels. Cellular sensitivity against DOX, paclitaxel, and colchicine were completely restored after shRNA expression, a result more successful than transient siRNA-mediated suppression reported by others. The P-gp was induced in these cells by a single-step selection after exposure to low DOX concentrations equivalent to that found in leukemia patients.[29] Unlike engineered cell lines with higher surface concentrations of P-gp, the native-like cells used in that study might have been more responsive to silencing due to lower concentrations of the transporters. However, even engineered cells with high P-gp levels were reported to respond well to shRNA-mediated silencing; for example, sensitivity of human colon carcinoma HCT-8 cells displayed increased sensitivity as IC_{50} decreased (based on IC_{50} assessment after vincristine treatment)[66] or multidrug-resistant gastric carcinoma EPG85-257RDB cells reverted completely to drug-sensitive phenotype (with daunorubicin) after stable shRNA expression.[31] In a similar approach, an MDR-1 shRNA was delivered to drug-resistant ovarian COC1/DDP and SKOV3/DDP cells (with Oligofectamine™), selected after stepwise selection in increasing concentration of cisplatin (DDP) from the parent cell lines. The P-gp mRNA was downregulated by 50–60%.[67] A combination of Oligo/Lipofectamine™ complex was similarly used to deliver a shRNA plasmid in several ovarian cancer cell lines (A2780, CAOV3, SKOV3, and SW626). While qualitative suppression at mRNA and protein levels were demonstrated, paclitaxel-induced apoptosis was increased by 2–3-fold in these cells.[68]

The innate ability of viral vectors to transfect human cells makes them alternative to physicochemical approaches to nucleic acid delivery. Adeno-associated viruses (AAV) have been attractive among the viral vectors, because they are able to transduce both dividing and nondividing cells with long-term expression.[69] The self-complementary recombinant AAV vector is suitable for short Pol III-based cassettes used for shRNA expression, and this vector was employed to express a MDR1 shRNA that were able to reduce P-gp levels after 2 days in the breast cancer NCI/ADR-RES cells. Knockdown of the P-gp mRNA was complete within 3 days and $>80\%$ reduction in P-gp protein level was obtained after 7 days. This led to an 8-fold increase in Rhodamine 123 (a nonpharmacological substrate of P-gp) uptake in transfected cells and a $\sim$2-fold increase in the DOX toxicity on transfected cells.[70] A self-inactivating retroviral expression vector was also employed to gain stable P-gp downregulation in drug-resistant cell line MES-SA/DX5 (established from human sarcoma MES-SA cells in the presence of increasing DOX).[71] The transfected cells were selected under hygromycin pressure, which yielded cells with high ($\sim$90%) level of P-gp downregulation after a 2-month transfection. Selection of the transfected cells by specific markers is an effective approach for obtaining high transfection efficiency in vitro; however, this is not likely to be a clinical reality.

A novel siRNA transfection system called the transkingdom RNAi has been also used to downregulate P-gp in the human gastric carcinoma cells EPG85-257RDB.[72] This delivery system uses nonpathogenic bacteria, such as *Escherichia coli*, to produce and deliver therapeutic shRNA into target cells. Using this method, 70% downregulation of P-gp mRNA was obtained based on real-time PCR analysis. Additionally, considerable suppression of P-gp protein was detected, and resistance to daunorubicin was reversed by 90%. Furthermore, anthracycline accumulation was considerably enhanced in the drug-resistant cancer cells.[72]

Finally, shRNA against P-gp was successfully expressed in drug-resistant, human hepatoma HepG2[73] and MaTu tumors[74] in nude mice. For HepG2 tumors, the expression vector for shRNA was mixed with a liposomal carrier (not specified) for intraperitoneal injection. Presumably, such an injection resulted in significant uptake of the expression system by tumor tissue. Indeed, a ~29% reduction in P-gp expressing cell population was seen after injection, indicating significant RNAi effect. The functional response to the chemotherapeutic drug adriamycin was also significant, where a ~58% reduction in tumor volume was seen after 2 weeks.[73] In MaTu tumors, intratumoral hydrodynamic injection of the shRNA expression vector resulted in 70–80% of tumor cells expressing the transgene, with a reduction of MDR1 levels to that of ~25% of the tumors.[74] Significant retardation of tumor growth (with DOX treatment) was observed over a 40-day study period, even though the P-gp protein suppression was relatively short lived (~8 days). These studies clearly demonstrated the feasibility of expressing exogenous shRNA for reversal of MDR in an animal model, but more studies will be needed to better understand shRNA expression at other sites because this might have significant impact in the overall physiology of an organism.

5. FUTURE AVENUES OF RESEARCH

An inspection of Table II should attest to the fact that most studies on P-gp silencing employed commercially available carriers for siRNA delivery. Although effective in siRNA delivery, the proprietary nature of the carriers impedes one to design superior delivery vehicles and, in particular, to tailor the carriers to meet the demands of cellular physiology in the context of target silencing and tumor targeting. One should be able to engineer the carrier properties to meet the needs of the individual cells, and this is only possible with the full knowledge of the carrier structure and formulation details. Our unpublished results have indicated large cell-to-cell variations in downregulation of common reporter genes with the same delivery system. For example, in one case, successful GAPDH downregulation in MDA-435/LCC6 MDR1 cells was obtained, but no effect was seen in MDA-MB-231 breast cancer cells with the same carrier/siRNA combination, despite equivalent quantitative delivery to both cell types (unpublished). In another case, an oleic acid-substituted PEI was effective in downregulating integrin α_v in B16 melanoma cells, but ineffective for P-gp downregulating in MDA-435/LCC6 MDR-1 cells.[57] A systematic approach to elucidate such differences requires further carrier optimizations, which is impossible to attempt without a well-defined starting point.

In tumors undergoing chemotherapy, upregulation of P-gp is usually accompanied with other changes in intracellular mediators. Antiapoptotic mediators, in particular, such as Bcl-xL,[75] mcl1,[76] and XIAB,[77] were reported to be upregulated in certain transformed cells, in addition to the P-gp. These mediators act by a variety of means to prevent apoptosis; for example, Bcl-xL prevents mitochondrial release of cytochrome C needed for activation of caspases, XIAB binds, and inactivates caspases 3, 7 and 9 needed for proteolysis of specific intracellular proteins, while mcl1 stabilizes mitochondrial membrane, preventing apoptosis initiation. Simultaneous inhibition of antiapoptotic mediators along with P-gp was recently

shown to be more potent in reversing the chemoresistance displayed by some cell lines,[76,77] so that increased potency can be achieved with dual siRNA delivery approach to coordinated targets. While P-gp activity could be linked to certain mediators, potentially leading to synergistic effect after dual silencing, other mediators might function independent of P-gp, so that a simple additive effect could be achieved with a dual delivery approach. The dual delivery has the potential to not only enhance the potency of chemotherapy, but also to restrict treatment to aberrant cells, rather than tissues displaying normal levels of the target molecules. Multiple siRNAs may lead to more specific effects than certain pharmacological agents that are shown to modulate a multitude of intracellular mediators, the spectrum of which might be difficult to control, leading to unpredictable actions by the latter pharmacological agents.[78]

Independent studies have overwhelmingly demonstrated the feasibility of P-gp downregulation using both nonviral carriers as well as viral vectors, but complete knockdown is not common. What happens to a tumor that retains a subpopulation of cells with P-gp activity is not clear. Will those cells display selective resistance to chemotherapy and take over the tumor tissue, ultimately creating a stronger MDR phenotype initially observed? Studies focusing on the reason for lack of complete P-gp downregulation (i.e., ineffective siRNA delivery/expression, inhibitory pathways for exogenous siRNA/shRNA, auxillary molecules supporting P-gp activity, upregulation of other transporters, etc.) will be needed to better understand this issue. This will be critical to develop a foundation to tackle any resistant tumor that might arise from silencing therapy against MDR. In a novel approach, N-linked glycosylation of P-gp (based on ribophorin II gene), have been introduced as a new target for MDR reversal. Interestingly, ribophorin II knockdown by siRNA has shown a stronger growth inhibitory effect compared to P-gp siRNA in drug-resistant breast cancer cells. This indicates an active role of ribophorin II-mediated P-gp glycosylation in P-gp-mediated MDR.[79]

Despite reasonable success gained in the reversal of MDR by siRNA-mediated P-gp knockdown, clinical trials on this process have not yet been attempted due to several limitations. These limitations are: (1) majority of the studies have been limited to cell culture studies, with only a few animal studies probed; (2) duration of the animal studies was limited to a maximum of 8 weeks and the effect of P-gp knockdown on the long term efficacy remains to be established, with better elucidation on survival rates in preclinical models; (3) animal studies typically involved intratumoral siRNA injection which is not realistic in humans. A systemic, tumor-targeting delivery of siRNA is needed for this reason, along with systemic administration of chemotherapeutic drug. Tumor targeting of siRNA is important as P-gp is found in other tissues with similar mRNA sequences and off-target knockdown will raise toxicity in these sites while decreasing the efficiency of the target knockdown (below). Local administration (i.e., nearby a tumor rather than intratumorlly) is a possible strategy but no work in this area has been reported, and; (4) many siRNA delivery systems may be inappropriate for human use, due to their ability to cause excessive inflammation and other adverse effects. It is important to address these critical issues before further progress in clinical trials of siRNA-mediated P-gp knockdown.

Finally, it was shown to be feasible to downregulate P-gp at other sites, such as blood–brain barrier[80] and liver,[73] after systemic injection of siRNA. Such an "undesirable" effect is bound to occur with siRNA delivery attempts to tumors. It will be important to develop a better sense of doses required for such off-target effects and discovering ways to minimize this undesired action. At least at the blood–brain barrier, repeated injection under "hydrodynamic" conditions were needed as well as excessive doses ($>50\,\mu g$/mice $\sim>2{,}500\,\mu g/kg$), so that the typical dose employed in tumor growth models ($\sim 15\,\mu g$/injection) might be low enough for avoiding side effects at other organs. More studies will be needed to

better characterize the P-gp response at other anatomical sites in preclinical studies to evaluate the side-effects of siRNA administration.

ACKNOWLEDGMENTS

The research was conducted at the authors' lab. We thank several past trainees in our labs for contributing to the development of the ideas presented in this work, in particular to Drs Vanessa Incani, Remant Bahadur K.C., Aws Alshamsan, Xiaobing Xiong and Azita Haddadi. We thank our colleagues Dr. Michael Weinfeld, Kamaljit Kaur, and Luc Berthiaume for their valuable insight on our siRNA delivery research.

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