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Delivery systems for the *MDR*1 gene

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Summary

The acquired resistance of cancer cells to a wide variety of structurally unrelated anti-cancer drugs as well as the increased sensitivity of cells of the hematopoietic system to these same drugs have contributed to the limited success of long term cancer chemotherapy. The overexpression of the multidrug resistance (*MDR*1) gene was found to be associated with this acquired resistance and has been exploited to protect normal bone marrow cells from myelosuppression that may result in life threatening leukopenia and thrombocytopenia following high dose chemotherapy. Thus far, retroviral transfer of the *MDR*1 gene has been the main route of delivery into bone marrow cells. Although widely used as one of the most efficient vehicles for gene delivery, safety and other concerns associated with viruses cannot be ignored. We have explored other means of introducing the *MDR*1 gene into recipient cells. A variety of vectors can be introduced into cultured cells and bone marrow cells *in vitro* using lipofection. One new system under development uses lipofection to introduce an Epstein Barr Virus (EBV)-based episomal vector carrying the *MDR*1 cDNA. High efficiency transfection of cultured cells has been achieved with this system.

I. Drug resistance in cancer

Metastatic and disseminated cancers that are not amenable to surgical removal or radiation can be treated by chemotherapy. Although many different malignances have been successfully treated by various antineoplastic drugs, the majority of solid tumors are either refractory to treatment or become non-responsive after an initial effect. Dose escalation to improve the efficacy of these chemotherapeutic agents is severely limited by their toxic side effects since normal tissues, especially the bone marrow, are particularly sensitive to anti-cancer agents.

The mechanism by which cancers evade chemotherapy has been intensively pursued. This has led to the elucidation of various cellular and genetic changes that confer increased drug resistance to cancer cells. These include decreased influx or increased extrusion of anti-cancer drugs; metabolic and cell-cycle changes in response to these drugs; augmented repair of drug-induced damage; to name a few (Gottesman et al., 1994). **Table 1** illustrates examples of gene products implicated in mediating resistance to anti-cancer drugs. Of these, the multidrug transporter, *MDR*1, has generated significant interest since it confers resistance to a wide variety of structurally unrelated drugs and has been found to be expressed at levels likely to contribute to drug resistance in 50% of metastatic and disseminated cancers (Gottesman and Pastan, 1997).

II. Structure of the multidrug transporter

The human *MDR*1 gene encodes a 170 kDa cell-surface phospho-glycoprotein known as P-glycoprotein ("P" also stands for permeability or pump). P-glycoprotein is an ATP-dependent efflux pump belonging to a superfamily of ATP-binding cassette (ABC) transporters. It is composed of two homologous halves, each spanning the plasma membrane six times and containing an ATP utilization site (**Fig. 1**). ATP binding and hydrolysis is important for substrate transport (Azzaria et al., 1989). Mutational analysis revealed three N-linked glycosylation sites present at the amino-terminal half in the first extracytoplasmic loop (Schinkel et al., 1993). These glycosylation sites may contribute to the correct

Figure 1. Hypothetical model of the human multidrug transporter, P-glycoprotein. Schematic model derived from sequence analysis depicting 12 transmembrane domains and 2 ATP sites. In this diagram, each circle represents an amino acid residue with filled in circles showing the positions of mutations that change the substrate specificity of P-glycoprotein. The squiggly lines are glycosylation sites. Known sites of phosphorylation are marked as (P) and the sites which are photoaffinity labelled are show as (| | |). The ATP sites are circled with the Walker A, B and C (linker dodecapeptide) regions indicated with capital letters. Adapted from Gottesman and Pastan (1988) J. Biol. Chem. 263, 12163-12166.

Table 1. Gene Products mediating resistance of anticancer drugs

DRUG RESISTANCE	GENE PRODUCT	REFERENCES
Methotrexate	Dihydrofolate reductase	O'Hare, *et al* . 1981 PNAS 78:1527-1531
Cyclophosphamide	Glutathione-S-Transferase π	Batist, *et al*. 1986 J. Biol. Chem. 261:15544-15549
Nitrosoureas	O^6 methylguanine methyltransferase	Hayakawa, *et al*. 1990 J. Mol. Biol. 213: 739-747
Mainly anionic-conjugated drugs	multidrug resistance protein (MRP)	Cole, *et al*. 1992 Science 258:1650-1654
	Cytochrome P450 3A	Wacher, *et al*. 1995 Mol. Carcinog. 13: 129-134
Structurally unrelated hydrophobic drugs, neutral and cationic	Multidrug Transporter (*MDR*1)	Gottesman, *et al*. 1995 Ann. Rev.Genet. 29: 607-649

Adapted from Gottesman *et al*. 1994 Annals of NY Acad Sci. 716: 126-139.

Table 2. Agents Interacting with the multidrug transporter

Anticancer Drugs
Vinca alkaloids e.g. vinblastine
Anthracyclines e.g. doxorubicin
Epipodophyllotoxins e.g. etoposide
Antibiotics e.g. actinomycin D
Others e.g. taxol, mitomycin C

Other Cytotoxic Drugs
Antimicrotubule drugs e.g. colchicine
Protein synthesis inhibitors e.g. puromycin
Toxic peptides e.g. valinomycin
DNA interchelators e.g. EtBr.

Reversing Agents
Calcium channel blockers e.g. verapamil
Anti-arrhythmics e.g. quinidine
Antihypertensives e.g. reserpine
Antibiotics e.g. hydrophobic cephalosporins
Antihistamines e.g. terfenadine
Immunosuppressants e.g. cyclosporin A
Steroid hormones e.g. progesterone
Modified Steroids e.g. tamoxifen
Lipophilic cations e.g. tetraphenylphosphonium
Diterpenes e.g. forskolin
Detergents e.g. Tween 80
Antidepressants e.g. tioperidone
Antipsychotics e.g. phenothiazines
HIV-1 protease inhibitors e.g. ritonavir
Other hydrophobic, amphipathic drugs and their analogs

Adapted from Gottesman and Pastan, 1993. Ann. Rev. Biochem. 62: 385-427.

folding, routing and/or stabilization of P-glycoprotein but does not seem to play a role in the drug efflux activity of P-glycoprotein (see (Gottesman et al., 1995; Schinkel et al., 1993)). Although sequence analysis revealed more than 40 consensus PKC and/or PKA phosphorylation sites distributed throughout the primary structure of the human P-glycoprotein, only very few (~4) are thought to be phosphorylated. Phosphorylation of these sites does not seem to play an essential role in drug transport (Germann et al., 1996).

Unlike other members of the ABC superfamily of transporters, the multidrug transporter can detect and extrude a wide spectrum of structurally and functionally diverse compounds (**Table 2**). These include various anticancer drugs like the vinca alkaloids, anthracyclines and epipodophyllotoxins as well as other cytotoxic agents. It is also capable of mediating transport of non-cytotoxic clinically useful pharmacological agents like calcium channel blockers (e.g. verapamil), immunosuppressive drugs (e.g. cyclosporine A), antihistamines, etc. These drugs or their analogues, also known as reversing agents or chemosensitizers, can serve as competitive inhibitors to block and sensitize the multidrug transporter to the anticancer drugs and reverse the multidrug resistance phenotype. An examination of the types of compounds that serve as *MDR*1 substrates reveal that they are generally hydrophobic and are usually positively charged at physiological pH (see (Gottesman and Pastan, 1993)). Both photoaffinity labelling and mutational analyses suggest that the transmembrane domains in the two halves of P-glycoprotein are important in determining the substrate specificity of the drug transport function. The fifth and sixth

transmembrane domains including the extracytoplasmic loop between them in the N-terminal half as well as the corresponding 11th and 12th transmembrane domains and their intervening loop at the C-terminal have been shown to be the major drug binding sites (see (Gottesman et al., 1995)).

III. Distribution and role of the multidrug transporter in normal tissues

An appreciation of the normal distribution and physiological role of *MDR*1 is important for the design and implementation of any intervention strategy. The multidrug transporter is expressed in a cell and tissue specific manner providing some hints as to the physiological role of this transporter. Some of these roles were recently confirmed when homologous genes in mice were inactivated using gene knockout technology (see (Borst and Schinkel, 1996)). Three major roles can be deduced from the major tissue distribution patterns of the multidrug transporter.

1. Role in steroid transport.

The plasma membrane of the adrenal cortex, site for steroid secretion, contains the highest concentration of *MDR*1. Another tissue involved in steroid secretion, the endometrium of gravid uterus and placenta also expresses *MDR*1. This suggests that the multidrug transporter may be involved either in the secretion process itself or protecting the membranes of secreting cells from the toxic effects of high concentration of steroids. Interestingly, *mdr*1(a/b) knockout mice are fully viable and have normal litters.

2. Role in transepithelial transport of endogenous metabolites and xenobiotics.

High concentrations of *MDR*1 can also be found in the luminal surfaces of epithelial tissues like the brush border of proximal renal tubules; mucosal surfaces of the large and small intestines as well as the apical surfaces of pancreatic ductules and biliary canalicular surface of hepatocytes. The localization of *MDR*1 in these tissues implies that the transporter may also be involved in the normal excretion of various endogenous metabolites or exogenous xenobiotics. This role is further suggested by the phenotype of the *mdr*1 knockout mice. These mice exhibited delayed clearance kinetics of the drug, vinblastine, which is consistent with a defect in either liver or kidney excretion of the drug (Schinkel et al., 1997; Schinkel et al., 1994; Schinkel et al., 1995).

3. Role in blood-brain, blood-germ cell/fetus barrier.

Expression of the *MDR*1 in the capillary endothelial cells of the brain, testis and placenta is suggestive that the multidrug transporter may be involved in keeping cytotoxic products out of the brain, germ cells and fetus. This role is supported by the observation that transgenic knockout mice lacking the mouse *mdr*1b gene accumulate toxic levels of the anticancer drug, vinblastine and the antihelminthic agent, ivermectin, in their brains (Schinkel et al., 1994; Schinkel et al., 1996)

Some expression of *MDR*1 can be found in human hematopoietic progenitor cells ($CD34^+$) (Chaudhary and Roninson, 1991) and peripheral blood including certain subpopulation of T cells (Chaudhary et al., 1992; Drach et al., 1992). Mature bone marrow cells expressed even lower levels of *MDR*1 suggesting a downregulation of this gene during maturation of the hematopoietic stem cells. Possible roles of the multidrug transporter in the hematopoietic stem cells include the export of a regulatory molecule to modulate their differentiation and proliferation and/or the protection of the stem cells against toxic insults.

IV. Protecting bone marrow cells from the toxic effects of chemotherapy

As mentioned earlier, *MDR*1-based multidrug resistance during chemotherapy can be circumvented either by the use of reversing agents, which may cause other side effects or by increasing the dose of the chemotherapeutic agent. However, high doses of chemotherapy causes myelotoxicity resulting in leukopenia, thrombocytopenia and anemia in the patient since the hematopoietic system is highly sensitive to anti-cancer drugs. One possible solution would be to introduce the *MDR*1 gene into the sensitive hematopoietic cells to protect these cells from anticancer agents. This has been demonstrated to be feasible in animal studies. A transgenic mouse model has been made in which the MDR1 cDNA driven by the chicken β-actin promoter was expressed in bone marrow cells (Galski et al., 1989). Normal bone marrow function was observed in these transgenic mice and they tolerated higher doses various anti-cancer drugs (Galski et al., 1989; Mickisch et al., 1992; Mickisch et al., 1991; Mickisch et al., 1991). Furthermore, bone marrow from these transgenic mice was successfully engrafted into lethally irradiated sensitive mice confering a multidrug resistant phenotype on the receipient mice (Mickisch et al., 1991). Retroviral transduction of the *MDR*1 cDNA in mouse bone marrow cells and subsequent introduction of these cells into irradiated sensitive mice also render the bone marrow of recipient mice drug resistant (Hanania and Deisseroth, 1994; Licht et al., 1995; Podda et al., 1992; Sorrentino et al., 1992). Several observations in these mice suggest that the *MDR*1 gene was in fact delivered into stem cells. These include long term expression of the multidrug transporter in different hematopoietic lineages, a single proviral integration site in different cells and persistence of gene expression following serial transplantation of bone marrow from resistant to sensitive mice. Greater drug tolerance was also observed in simian models transduced with the *MDR*1 gene (Boesen et al., 1995). The multidrug transporter gene has also been introducted into human hematopoietic cells via retroviral transduction (Hegewisch-Becker et al., 1995). Besides bone marrow, various other sources of hematopoietic stem cells can be used to introduce the *MDR*1, including cord blood (Bertolini et al., 1994; Williams and Moritz, 1994) and mobilized peripheral blood progenitors (Chen et al., 1995; Prosper et al., 1997; Scott et al., 1997; Sekhsaria et al., 1993; Sutherland et al., 1995). Several clinical trials are being performed to evaluate the feasibility of dose escalation in advanced cancer patients receiving bone marrow that has been transduced with *MDR*1 retroviruses (Deisseroth et al., 1994; Hesdorffer et al., 1994; O'Shaughnessy et al., 1994; Rosenberg et al., 1996).

V. Retroviral vs non-viral delivery

Successful gene transfer is dependent on two important steps, namely efficient delivery of the transgene to the appropriate cells and its subsequent maintenance and expression. Delivery modalities can be viral or non-viral. The most exploited system for gene transfer is via murine retroviral vectors (Miller, 1992; Miller et al., 1993; Miller and Rosman, 1989). These vectors have thus far been the main route of *MDR*1 gene transfer into hematopoietic cells. They are replication-defective with their "non-essential genes" deleted and replaced with the gene of interest. The "non-essential genes" are then supplied in trans since they encode proteins that are important for viral functions incuding replication and packaging. Gene transfer is effected by the binding of the vector to receptors on the target cells. The advantages of the retroviral system are that they are relatively safe and efficient, their host range can be manipulated, and they can stably integrate into the host chromosome for persistent expression. Disadvantages of these vectors include the requirement of murine retroviruses for active DNA replication/cell division and their propensity to integrate randomly into the host genome increasing the risk for insertional mutagenesis. Another safety concern is the generation of unpredictable replication competent viruses via recombination of the viral vector sequences with endogenous or exogenous helper viruses. Hence, non-viral modalities of gene delivery as a plausible alternative has been given increasing attention.

A popular non-viral approach to gene transfer is liposome-mediated delivery. Entry of naked DNA into a cell is hindered by the size of the DNA which is typically in the micron range as well as the similar charge on the DNA and the cell membrane causing them to repel each other. Liposomal delivery systems serve to compact the DNA and provide a "double-sided sticky tape" to bind the anionic DNA to the anionic cell membrane so as to favor membrane destabilization or endocytosis. Basically, polycationic lipids are mixed with plasmid DNA to form liposomes which will then fuse with the target cell and mediate gene transfer (Felgner and Ringold, 1989). Different formulations of lipids have been developed. These usually consist of mixtures of a neutral co-lipid exhibiting fusogenic properties with a cationic lipid or cytofectin to form cationic liposomes which are then mixed with DNA before being introduced into cells.

The formulation we have used was first described by Behr (Behr, 1986) and refined by Thierry et al. (see (Thierry et al., 1997)). These are lipopolyamines (DLS) consisting of dioctadecylamidoglycylspermine (DOGS) (Transfectam™; Promega) and Dioleylphosphatidyl ethanolamine (DOPE) (see **Figure 2**) with a self-aggregating hydrocarbon tail linked to a polycationic DNA-compacting headgroup. It has protonatable polyamines as lipid head groups which can buffer the complex against endosomal degradation and effectively condense DNA into a discrete DNA-lipopolyamine complex in the nanomicron range and has been found to be relatively non-toxic.

One advantage of this gene delivery system is that since liposomes are mainly made up of DNA and lipids and contain no proteins, host response is minimized. Liposomes can also potentially accomodate larger sized DNA. Furthermore, liposome-DNA complexes are technically simpler to prepare, test and scale-up compared to retroviral vectors. Due to their inherent modularity, they can be designed to target different tissues. However, for this delivery system to be useful for clinical gene therapy, a number of obstacles must be surmounted. Compared to viral vectors, this method of introducing DNA into cells is relatively inefficient (Baudard et al., 1996). Moreover, targeted gene delivery using conventional liposomes is limited by the selective uptake of liposome-DNA complexes by cells of the reticuloendothelial system (RES). This can be partially circumvented by the use of long-circulating, stearically stabilized or "stealth" liposomes which display monosialoganglioside GM1 or polyethylene glycol (PEG) (Allen, 1994).

Figure 2: Structure of lipopolyamines, DLS. **Top panel**: Dioctadecylamidoglycylspermine or DOGS. **Bottom panel**: Diolelylphosphatidyl ethanolamine or DOPE.

VI. Maintenance of the transgene within cells

Maintenance of the transgene can be achieved either through the integration of the transferred DNA into the host genome or as an autonomously replicating extrachromosomal element or episome. Retroviruses have evolved effective ways to mediate integration of their reversed transcribed DNA into the host genome. However, integration of a random supercoiled DNA into the genome occurs only occasionally. Hence, the addition of sequences that allow episomal replication may be advantageous. Vectors that replicate episomally can be created by incorporating the origin of replication and a gene product important for maintaining the episomal replication from either the Epstein

Barr Virus (EBV) virus (Margolskee, 1992; Sabbioni et al., 1995) or the BKV vectors (Sabbioni et al., 1995; Thierry et al., 1995). These vectors can maintain high levels of expression via vector amplification. EBV episomes have been found to replicate in lymphoid cells at ~10-50 copies per cell, while BK virus can replicate in diverse cell types into ~150 copies per cell. Maintaining the transgene episomally is attractive as it reduces the risk of insertional mutagenesis as well as host cis-chromosomal effects on the transgene expression.

The episomal vector that was utilized in our studies is derived from EBV, a human lymphotropic herpesvirus (see (Margolskee, 1992)). Its genome is 172 kb long and encodes approximately 100 different genes, most of which are responsible for viral production. The life cycle of EBV comprises two phases, a lytic and a latent phase (**Fig 3**). More than 90% of the adult human population harbors this virus in its latent phase asymptomatically. The virus exists latently as an episome. The episomal phase of EBV is maintained by two elements interacting to ensure that the viral genome is retained within the nucleus, efficiently replicated and properly partitioned into daughter cells. Replication is bidirectional occuring once per cell cycle in synchrony with the host. The cis-acting element is the episomal origin of replication, known as OriP. The OriP **(Fig 4**) maps to approximately 1.8 kb of the EBV genome and comprises two distinct sets of sequence motifs, both of which are important for replication. They are the family of repeats (FR) comprising 20 tandem repeats of 30 bp in length each and the dyad symmetry (DS) component which is approximately 140 bp in length and contains a 65 bp dyad symmetry. The FR is separated from the DS by about 960 bp of sequences. Replication is initiated at the DS while the FR serves as a replication fork barrier. The transacting element responsible for episomal maintenance of EBV is the EBV nuclear antigen 1 (EBNA-1). EBNA-1 is a 65-80 kDa phosphoprotein that is encoded by a 2 kb open reading frame (ORF) within a 3.7 kb transcript. This protein comprises unique N and C-terminal domains joined by a central domain that contains glycine-alanine (G-A) repeats. These G-A repeats were found not to be essential for the transacting functions of EBNA-1 (Yates et al., 1985) but were recently implicated in viral escape from the cytotoxic T lymphocyte (CTL) surveillance (Levitskaya et al., 1995). EBNA-1 plays an important role in replication as well as transcriptional activation by binding to the FR and related sequences in DS. Interaction of EBNA-1 with DS initiates bidirectional replication while binding of EBNA-1 to FR enhances transcription from the episome and terminate DNA replication. These two elements have been widely exploited to maintain other genes of interest episomally.

To ensure long term expression of the transgene in this episomal configuration in rapidly dividing cells, selective pressure has to be applied. An episomally maintained *MDR*1 that is introduced into rapidly dividing hematopoietic stem cells would be very useful to protect hematopoietic cells

Figure 3: Life cycle of EBV.

Figure 4: Structure of OriP.

against the toxic effects of high dose chemotherapy. This is because the drugs that are used to kill cancer cells during chemotherapy will be the same drugs that will maintain the *MDR*1 gene in the sensitive hematopoietic cells at relatively high copy numbers preventing the toxic drugs from entering these cells.

VII. Liposome delivery of an episomal vector containing *mdr*1

To test the feasibility of utilizing an episomally maintained *MDR*1 for gene therapy to protect normal hematopoietic cells against the toxic chemotherapeutic drugs, an OriP/EBNA containing construct was made (see **Fig 5**). This construct (pEBV-Ha*MDR*1) is derived from the parental pHa*MDR*1 and is similar in every respect to pHa*MDR*1 except for the presence of OriP/EBNA from EBV and the hygromycin gene driven by the thymidine kinase promoter. The expression of *MDR*1 in this construct is also driven by the Harvey sarcoma retroviral promoter. After transfection into cultured cells, the EBV-Ha*MDR*1 vector system gives a high frequency of drug resistance and the resistant cells have high levels of P-glycoprotein on their surfaces as detected by FACS analysis with a monoclonal antibody specific for

Figure 5: Structure of OriP/EBNA-1 containing construct, pEBV-Ha*MDR*1 (left panel) and its parental construct, pHa*MDR*1 (right panel)

human P-glycoprotein (Lee et al., unpublished data). The resistant cells can be maintained in selective medium for several months and extrachromosomal DNA could be recovered from HIRT supernatants (Hirt, 1967).

VIII. Other potential applications of liposome delivered, episomally maintained *mdr*1.

Besides its utility in protecting sensitive bone marrow cells from toxic chemotherapeutic drugs, such a vector can also be very useful in various other gene therapy applications. For an episomal vector to be clinically useful, an appropriate selectable marker is essential to maintain selective pressure for stable expression transgene. Although neo-resistance due to expression of a neomycin phosphotransferase DNA has been popularly used *in vitro*, its applicability *in vivo* is limited because of the toxicity of G418 (Valera et al., 1994). *MDR*1, in contrast, is a clinically relevant *in vivo* selectable marker. Surface expression of *MDR*1 allows for easy detection and sorting of cells containing this transgene. Mutations in different regions of the *MDR*1 gene change the relative resistance to different drugs, making it possible to construct "designer" *MDR*1 gene that distinguish cells containing the transgene from the endogenous gene (Gottesman et al., 1995). Empirically, *MDR*1 has been successful as a dominant selectable marker for the coexpression of many genes (see (Sugimoto et al., 1996)) including HSV-TK (Sugimoto et al., 1994; Sugimoto et al., 1995), glucocerebrosidase (Aran et al., 1996; Aran et al., 1994; Aran et al., 1996), α-galactosidase (Sugimoto et al.,

1995), a subunit of the phox flavocytochrome b_{558}, $gp91^{phox}$ (Sokolic et al., 1996) and ribozyme targetted to the U5 region of HIV1 (Lee et al., 1997).

In summary, although retroviruses have been the dominant method of gene transfer of *MDR*1 for chemoprotection, the usefulness of retroviral vectors may be limited by safety concerns. This chapter discusses another alternative for the transfer of *MDR*1 genes using liposome delivery. Long-term expression is maintained episomally using the EBV OriP/EBNA. Higher expression of the gene can be obtained using such a vector as more copies of the gene are maintained within the cell. Such a vector system combining liposome delivery, episomal maintenance, and *MDR*1 as clinically useful selectable marker, have potential applications for gene therapy.

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