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DEVELOPMENT AND CHARACTERIZATION OF P-GLYCOPROTEIN SPECIFIC MULTIDRUG RESISTANCE MODULATORS

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ABSTRACT

Multidrug resistance (MDR) is a phenomenon by which tumor cells develop reduced sensitivity to anticancer drugs, which often leads to the failure of cancer chemotherapy. A prominent mechanism of MDR is the overexpression of the multidrug efflux pump, P-glycoprotein (P-gp), which decreases the intracellular accumulation of many anticancer drugs leading to increased tumor growth. Intensive efforts are underway to develop clinically useful MDR modulators that inhibit the function of P-gp for use in combination with established anticancer drugs. To study the *in vivo* systemic effects of P-gp-specific inhibitors on reversing P-gp-mediated MDR, we developed a solid tumor model utilizing immunocompetent animals. Using *in vitro* cytotoxicity and drug accumulation assays, two transformed murine cell lines, JC and TIB-75, were found to demonstrate the P-gp-mediated MDR phenotype. In contrast, two similar lines did not express functional P-gp. Western blot analyses confirmed the expression of P-gp and the lack of expression of the closely related drug efflux protein MRP1 in the JC and TIB-75 cell lines. The JC cell line displayed excellent tumorgenicity and consistent growth kinetics when implanted into immunocompetent Balb/c mice. Animals treated with a combination of a known MDR modulator, cyclosporin A, and a cytotoxic drug, doxorubicin, exhibited significantly reduced tumor growth compared to untreated controls or animals treated with either cyclosporin A or doxorubicin alone. Therefore, this syngeneic solid tumor model provides a new *in vivo* system that can be used to evaluate the efficacy of P-gp inhibitors in an immunocompetent host and should provide improved prediction of the clinical utility of these compounds.

In our search for improved MDR modulators, we identified a novel series of substituted pyrroloquinolines that selectively inhibits the function of P-gp without modulating multidrug resistance-related protein 1 (MRP1). These compounds were evaluated for their toxicity towards drug sensitive tumor cells (i.e. MCF-7, T24) and for their ability to antagonize P-gp-mediated drug resistant cells (i.e. NCI/ADR) and MRP1-mediated resistant cells (i.e. MCF-7/VP). The *in vitro* cytotoxicity and drug accumulation assays demonstrated that the dihydropyrroloquinoline analogs inhibit P-gp to varying degrees without any significant inhibition of MRP1. One of the analogs, PGP-4008, showed the highest level of P-gp inhibition (P-gp antagonism score ≈ 17) *in vitro* and was further evaluated *in vivo*. PGP-4008 inhibited tumor growth in the JC murine syngeneic P-gp-mediated MDR solid tumor model when given in combination with doxorubicin. The dose of PGP-4008 (100 mg/kg) and the route of administration (intraperitoneal) used in the tumor model resulted in rapid systemic absorption with plasma concentrations exceeding the *in vitro* effective dose (0.8 μg/ml) for 2 h after administration. PGP-4008 did not alter the plasma distribution of concomitantly administered anticancer drugs. Furthermore, signs of systemic toxicity were not seen with the P-gp selective modulator, PGP-4008, as seen in comparison with a non-selective inhibitor, cyclosporin A. Because of their transport-selectivity, these substituted dihydropyrroloquinolines may be more effective MDR modulators than non-specific modulators in a clinical setting.

We also identified a series of quinoxalinones that could reverse MDR through the inhibition of P-gp, thereby increasing the efficacy of anticancer drugs in P-gp-expressing tumor cells. One compound in this series, 2-benzyloxy-3-methyl-quinoxaline (termed

BMQ), displayed a surprising result when administered with actinomycin D. Actinomycin D is a peptide-containing antitumor drug isolated from *Streptomyces* that inhibits RNA synthesis by intercalating into DNA. It is currently thought that actinomycin D enters cells by passive diffusion through the plasma membrane. However, studies described herein demonstrate that actinomycin D uptake by cells is saturable, temperature-dependent and energy-dependent, suggesting an active transport mechanism. BMQ markedly increased the accumulation of actinomycin D within cells overexpressing P-gp, as well as a variety of cell lines that do not express this drug efflux protein. This increased accumulation of [^{3}H]actinomycin D by BMQ was not due exclusively to the inhibition of efflux of the drug from the cell. Treating the cells with antimycin A, 2-deoxyglucose and sodium azide, which depleted intracellular levels of ATP by at least 75%, abolished the effects of BMQ on the cellular accumulation of actinomycin D. In addition, decreasing the temperature from 37˚C to 4˚C blocked the effects of BMQ. These studies suggest that the facilitation of actinomycin D transport by BMQ is not due to inhibition of efflux, and that actinomycin D transport is energy-dependent, temperature-dependent, and saturable. Overall, these studies suggest that a membrane transporter is responsible for the passage of actinomycin D across the cellular membrane, and that BMQ can potentiate this influx mechanism.

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[^{3}H]	tritium
ABC	ATP-binding cassette
ALL	acute lymphoblastic leukemia
AML	acute myelogenous leukemia
ATP	adenosine triphosphate
AUC	area under the curve
BCRP	breast cancer resistance protein
Bmax	maximum binding
BMQ	2-benzyloxy-3-methyl-quinoxaline
˚C	celsius
Ci	curie
CO_2	carbon dioxide
CsA	cyclosporin A
CV	coefficients of variation
DMEM	Dulbecco's modified eagle's media
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
F	bioavailability
FBS	fetal bovine serum
FK-506	tacrolimus
h	hour

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HPLC	high-performance liquid chromatography
IC	inhibitory concentration
i.e.	that is
IgG	immunoglobulin G
i.v.	intravenous
Kd	affinity constant
kDa	kilodaltons
kg	kilogram
LRP	lung resistance protein
LY335979	zosuquidar
µCi	microcurie
MDR	multiple drug resistance or multidrug resistance
µg	microgram
mg	milligram
µl	microliter
ml	milliliter
min	minute
µm	micrometer
mm	millimeter
µM	micromolar
mM	millimolar

mmol	millimoles
mp	melting point
mRNA	messenger ribonucleic acid
MRP	multidrug resistance-related protein
nm	nanometer
nM	nanomolar
NMR	nuclear magnetic resonance
NSCLC	non-small-cell lung cancer
P-gp	P-glycoprotein
PGP-4008	N-(1-Benzyl-2,3-dihydro-1H-pyrrolo[2,3-b]quinolin-4-yl)-2-phenyl-acetamide
PBS	phosphate-buffered saline
PKC	protein kinase C
p.o.	oral
PSC833	valspodar
PVDF	polyvinylidene fluoride
QSAR	quantitative structure-activity relationships
RI	reversal index
RNA	ribonucleic acid
RPMI	tissue culture media from the Roswell Park Memorial Institute
SCLC	small-cell lung cancer
SD	standard deviation
SDS	sodium dodecyl sulfate

SEM	standard error measurement
SNP	single nucleotide polymorphism
SRB	sulforhodamine B
ssDNA	single-stranded deoxyribonucleic acid
TMS	tetramethylsilane
VAC	vincristine, actinomycin D, cyclophosphamide
VX-710	biricodar
XR9576	tariquidar
YB-1	Y-box binding protein

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CHAPTER 1

LITERATURE REVIEW

A. Introduction

Cancer is the second leading cause of death in the United States today. Each year, there are over 1,000,000 people diagnosed with some form of cancer in the United States. Treatment for this disease involves surgery, radiation and/or chemotherapy. Chemotherapy regimens have become the standard of care for many different types of cancer with promising results. The regimen usually involves the use of drugs that inhibit the growth of the tumor. However, there are cases where patients do not respond to chemotherapy or develop refractory tumors that no longer respond to chemotherapy and become drug resistant.

There are two major classes of cellular resistance to anticancer drugs. In the first class of resistance, there are the factors within the tumor cell that change to affect its drug sensitivity. These mechanisms include increased expression of DNA repair enzymes, defective apoptotic pathways (i.e. p53 mutations), and altering the cellular target of the drug. The second class of resistance is when the delivery of the drug to the cell is inhibited. Mechanisms for this method of resistance include increased efflux of drug (i.e. expression of ATP-dependent efflux pumps), decreased uptake of drug into the cell, increased metabolism of drugs (i.e. P450 enzymes, glutathione-S-transferase), and altered drug distribution (Gottesman et al., 2002).

Multidrug resistance (MDR) is a phenomenon that leads to the failure of chemotherapy in cancer patients by allowing tumors to intrinsically resist or acquire resistance to a variety of structurally and mechanistically unrelated anticancer drugs. This drug resistance often results from elevated expression of particular proteins such as cell-membrane transporters that function by increasing the efflux of cytotoxic drugs from cancer cells. This allows the cancer cells to survive by lowering the intracellular concentrations of drugs. The cytotoxic drugs that are most frequently associated with MDR are hydrophobic, amphipathic natural products including taxanes (paclitaxel, docetaxel), vinca alkaloids (vinblastine, vincristine), anthracyclines (doxorubicin, daunorubicin), epipodophyllotoxins (etoposide), actinomycin D, and mitomycin C (Ambudkar et al., 1999).

Over the past 15 years, there has been a major effort in clinical oncology to develop modulators/inhibitors that can effectively reverse the MDR phenotype, thereby making those resistant tumors no longer resistant. This change in phenotype is expected to allow for the treatment of the tumor and lead to increased long-term survival for the patients. A major mechanism of this phenotype is the overexpression of the energy-dependent, transmembrane efflux pump, P-glycoprotein (P-gp). In recent years, there has been a concerted effort in the development of specific P-gp modulators that are more potent and possibly more efficacious than their predecessors. The goal of this review is to establish the rationale for the development of P-gp specific MDR modulators and to describe how the specificity will contribute to increased clinical efficacy.

B. ATP-dependent transporters as a mechanism of MDR

A major mechanism of MDR is the overexpression of energy-dependent, unidirectional transmembrane efflux pumps that belong to the ATP-binding cassette (ABC) transporter family. The ABC transporters include P-gp transporters (MDR1 and MDR3 genes in human), the MRP subfamily and other proteins, including the breast cancer resistance protein (BCRP). Currently, there are a total of 48 human ABC genes identified, which have been divided into seven subfamilies (ABCA-ABCG) based on their domain organization and sequence homology (Dean et al., 2001).

The drug transporter encoded by the MDR1 gene, P-gp (ABCB1 subfamily), is a 170 kDa protein product that was first discovered in 1976 (Juliano and Ling, 1976). Its biochemistry and pharmacology has been intensely studied for over 25 years (Ling, 1997; Sikic et al., 1997; Tan, 2000). P-gp consists of two homologous and symmetrical halves that make up the cassettes in which each half contains six transmembrane domains separated by an intracellular polypeptide linking with an ATP-binding site (Gottesman and Pastan, 1993). As is common with ABC transporters, P-gp can recognize and transport a wide variety of diverse substrates that vary in structure, size and pharmacological effects. Table 1.1 lists a summary of substrates, including anticancer agents such as doxorubicin, vinblastine, paclitaxel and etoposide (Alvarez et al., 1995; Gottesman et al., 2002), as well as a number of other drugs with different pharmacological properties.

Drug Class	Substrates
Anticancer agents	actinomycin D, etoposide, paclitaxel, docetaxel, doxorubicin, daunorubicin, vinblastine, vincristine, irinotecan, topotecan, mitoxantrone, mitomycin C
Calcium blocker and metabolites	verapamil, diltiazem, nicardipine, mibefradil
Cardiac drugs	digoxin, digitoxin, quinidine
Steroids	aldosterone, dexamethasone, estradiol, hydrocortisone
Immunosuppressants	cyclosporin A, tacrolimus, rapamycin
Antibiotics	erythromycin, levofloxacin, sparfloxacin
HIV protease inhibitors	amprenavir, indinavir, nelfinavir, saqunavir, ritonavir
Lipid-lowering agents	atorvastatin, lovastatin
Others	debrisoquine, losartan, morphine, rifampin, phenytoin, rhodamine 123, triton X-100, halofantrine, amitriptyline

Table 1.1. Summary of P-glycoprotein substrates. Adapted from Sakaeda et al., 2002.

In vitro studies have shown that the expression of the MDR1 gene can be rapidly and transiently induced in cultured cell lines that have been acutely exposed to P-gp substrates, including doxorubicin (Chin et al., 1990; Chaudhary and Roninson, 1993; Hu et al., 1995; Tomida et al., 1995). This shows that the expression of P-gp is not constant and can be induced, and demonstrates a possible factor why refractory tumors have a higher incidence of P-gp expression than primary tumors.

A series of homologous proteins, termed multidrug resistance-related proteins (MRPs), have been discovered recently. These proteins share many pharmacological characteristics with P-gp (Grant et al., 1994; Kruh et al., 1994). The first protein of this series, MRP1 (ABCC1 subfamily), is a 190 kDa protein that was first discovered in 1992 from a resistant lung cancer cell line that did not express P-gp (Cole et al., 1992). MRP1 is similar to P-gp in structure except for an additional five transmembrane domains (Ambudkar et al., 1999). MRP1 typically recognizes neutral and anionic hydrophobic substrates, including vincristine, doxorubicin, and etoposide, and transports glutathione (Muller et al., 1994) and glutathione-conjugated drugs (Jedlitschky et al., 1996).

Other transporters that have been associated with drug resistance include BCRP (ABCG2 subfamily), a homodimer of two half-transporters, each containing six transmembrane domains and an ATP-binding site (Doyle et al., 1998), and MDR3, a protein closely related to MDR1, that functions as a phosphatidylcholine flippase that normally transports phospholipids into bile but can transport drugs such as paclitaxel and vinblastine (Ruetz and Gros, 1994; Zhou et al., 1999). Lung resistance protein (LRP), while not a member of the ABC transporter family, is another protein associated with

drug resistance that can be highly expressed in drug-resistant cell lines and some tumors (Scheffer et al., 2000b).

Many of these transporters recognize a variety of substrates and function by effluxing these drugs from the cell into the extracellular milieu. The transmembrane regions of the transporters bind preferentially to hydrophobic, amphipathic substrates which may contribute to the mechanism of action of removing substrates directly from the lipid bilayer by presenting them to the pump directly before the substrates reach the cytosol (Ambudkar et al., 1999). The transporters release the substrates into the extracellular space using energy from the hydrolysis of ATP (Horio et al., 1988). Transport of each drug molecule requires two ATP hydrolysis events that do not occur simultaneously (Senior and Bhagat, 1998). Tumor cells that are exposed to cytotoxic compounds often overexpress these efflux pumps, which allows them to survive when treated with anticancer drugs by reducing the intracellular accumulation. This removal of the anticancer drugs spares the tumor cells from the effects of the drugs by not allowing them to interact with their intracellular targets (Kartner et al., 1983).

There is evidence that MDR is usually multifactorial, with at least two resistance mechanisms that occur within the same tumor cell (Larsen and Skladanowski, 1998). P-gp and cytochrome P450 3A have overlapping substrate specificities and similar induction mechanisms. Therefore, it is not surprising that the induction of P-gp and cytochrome P450 3A have been shown to occur simultaneously in human colon carcinoma cells exposed to modulators and substrates of both enzymes (Schuetz et al., 1996). Furthermore, cancer cells that have been exposed to chemotherapeutic agents are likely to be genetically heterogenous because of the many different mechanisms by which

the cells can acquire resistance. Although any one mechanism may be sufficient for a tumor cell to develop resistance and survive, the mutation phenotype characteristic of tumors suggests that more than one single mechanism may be involved in the MDR phenomenon (Gottesman et al., 2002). The subsequent sections of the review will focus on the two most studied and characterized ATP-dependent transporters, P-gp and MRP1.

C. Tissue distribution and function of P-gp and MRP1

The ATP-dependent transporters P-gp and MRP1 have been identified through the use of drug-resistant cell lines and are associated with the MDR phenotype. However, these transporters are also expressed in normal tissues and transport specific endogenous substrates in addition to exogenously administered drugs and xenobiotics.

P-gp is expressed in many different normal tissues. Previous studies have shown that P-gp is expressed by certain types of secretory cells, including the capillary endothelial cells of the brain and testis and placenta. Its functional arrangement and location on the luminal surface allows the transport of xenobiotics from the organ into the bloodstream, thereby protecting and detoxifying these "sanctuary sites" and establishing the blood-brain, blood-testis, and placental barrier. P-gp is also expressed in the intestine, liver and kidney, allowing these organs to facilitate xenobiotics clearance and protect the entire organism. Other sites of P-gp expression include lymphocytes, adrenal glands and pancreas (Thiebaut et al., 1987; Endicott and Ling, 1989; Leveille-Webster and Arias, 1995; Lum and Gosland, 1995; Brinkmann et al., 2001).

Much of the understanding for the normal physiological role of P-gp comes from studying mice genetically deficient in the genes encoding for this transport protein and observing their phenotype with the administration of xenobiotics. Mice have two mdr1 genes (mdr1a and mdr1b) which are homologs of the human MDR1 gene. The mdr1a/1b (-/-) knockout mice develop normally but are extremely sensitive to certain xenobiotics and display strong alteration in the pharmacokinetics of P-gp substrate drugs. The oral bioavailability of anticancer drugs such as paclitaxel is markedly increased in the P-gp-deficient mice compared to wild-type (Sparreboom et al., 1997). Furthermore, the

deletion of endogenous P-gp allows for P-gp substrates that are normally denied entrance to the brain to pass through the blood-brain barrier (Schinkel et al., 1996; Schinkel et al., 1997). These genetic and pharmacokinetic studies support the physiological role of P-gp in protection and detoxification of the body.

By comparison, mRNA of MRP1 has been observed in virtually every type of tissue within the body (Zaman et al., 1993) and is expressed in high concentrations in peripheral blood mononuclear cells (Cole et al., 1992; Abbaszadegan et al., 1994; Burger et al., 1994; Hart et al., 1994; Schneider et al., 1995). MRP1 and other members of the MRP subfamily have also been shown to have a protective function (Scheffer et al., 2000a; St-Pierre et al., 2000). However, in certain tissues such as the intestine, MRP1 has been shown to have the opposite effect of P-gp, allowing the transport of substrates from the intestinal lumen into the bloodstream and thereby facilitating absorption rather than excretion (Evers et al., 1996).

The difference in tissue expression of the two transporters, P-gp and MRP1, has led to a new insight in the drug development of MDR modulators. Since the tissue distribution of MRP1 is much more ubiquitous in comparison to P-gp, it seems more likely that modulation of both P-gp and MRP1 would cause increased non-specific toxicity compared to modulation of just P-gp.

D. Gene expression of MDR1

An underlying cause of MDR is the increase in gene expression from gene amplification or transcriptional activation that results in increased expression of particular proteins that confer an advantage to tumor cells when treated with cytotoxic drugs. Increased expression of MDR1 mRNA is a common mechanism of MDR in human cells. There are two major mechanisms involved in the increased expression of MDR1: gene activation and gene amplification.

Activation of the MDR1 gene by mutations or epigenetic changes may precede its amplification during the development of the MDR phenotype (Shen et al., 1986). Gene rearrangements at the 5'-flanking region of the MDR1 gene cause gene activation in human lymphomas and MDR breast and colon cancer cells (Mickley et al., 1997). In the doxorubicin-selected human adenocarcinoma cell line, S48-3s, the MDR1 gene is activated after a 4;7 translocation of the gene that results in its repositioning 3' to a transcriptionally active chromosome 4 gene that has a higher endogenous expression than MDR1 normally does (Mickley et al., 1997).

The MDR1 gene has also been shown to be activated after exogenous stimulation from cytotoxic drugs and/or carcinogens (Fairchild et al., 1987b; Kohno et al., 1989; Mickley et al., 1989). This activation appears to be mediated through nuclear transcription factors including YB-1 (Y-box binding protein), where studies have shown that it has a role in transcriptional activation of the MDR1 gene in the presence of a genotoxic or environmental stress (Asakuno et al., 1994; Ohga et al., 1998).

The second mechanism is the frequent amplification of the MDR1 gene (Riordan et al., 1985; Torigoe et al., 1995). This gene amplification can be regulated by the

methylation of the promoter. DNA methylation has been shown to be a plausible regulator of gene expression since there is an inverse correlation between methylation and transcription in both normal and malignant cells (Boyes and Bird, 1991; Laird and Jaenisch, 1994). The regulation of the MDR1 gene does not seem to be immune to this correlation between methylation and transcription. Methylation of 5'CpG sites within the promoter region of the MDR1 gene negatively regulates its expression and the P-gp-mediated MDR phenotype in human cancer cell lines (Kantharidis et al., 1997; Kusaba et al., 1999) as well as clinical samples from acute myeloid leukemia (Nakayama et al., 1998) and bladder cancer patients (Tada et al., 2000).

These findings suggest that the increased expression of the MDR1 gene that leads to overexpression of P-gp and a MDR phenotype in some human cancers is induced by gene activation through rearrangement or localization of transcription factors such as YB-1 or by gene amplification through methylation of the MDR1 promoter.

Mutations within the MDR1 gene have been shown to exhibit varying degrees of functional protein depending on the location of the mutation. These mutants fall into three general functional categories: misprocessed biosynthetic mutants, mutants that affect substrate specificity and mutants that diminish the activity of the transporter. Mutations that affect substrate specificity generally are clustered in the transmembrane domains, especially in regions 5, 6, 11, and 12, which suggests that these regions are important for drug binding (Ambudkar et al., 1999).

Genetic polymorphisms of human MDR1 was first observed in a population of cancer patients, healthy volunteers and drug-resistant tumor cell lines. Single nucleotide polymorphisms (SNPs) were seen in exons 21 and 24 (G2677T and G2995A) (Mickley et

al., 1998). This led to the systemic screening of the entire MDR1 gene for the presence of SNPs. All 28 exons and the core promoter region were analyzed in 188 Caucasian individuals. A total of 15 SNPs were detected, which are listed in Table 1 (Hoffmeyer et al., 2000). The polymorphism in exon 26 (C3435T), which caused no amino acid change, had a profound effect on the expression levels of P-gp in the intestine. The T-allele, particularly if homozygous, is associated with low intestinal expression of P-gp. The corresponding C-allele is associated with increased P-gp expression. This differential expression can influence the uptake of orally administered P-gp substrates such as digoxin where individuals carrying the homozygous low-expressing T-allelle have higher concentrations of drug in the plasma due to increased uptake (Brinkmann et al., 2001). Homozygous individuals with the G2677T SNP in exon 21 show an increased dose requirement of tacrolimus (40% more) in comparison to wild-type patients. Genotype monitoring of the MDR1 gene has been shown to reliably predict the optimal dose for tacrolimus in renal transplant patients and can be used to predict the initial daily doses required to obtain adequate immunosuppression (Anglicheau et al., 2003).

The discovery of genetic variations within the MDR1 gene that influence the function or expression of P-gp can have a direct impact on the therapeutic efficacy of substrates as a result of the alteration in pharmacokinetics and pharmacodynamics. These polymorphisms within the human population can affect the likelihood of intestinal absorption or elimination of orally administered drugs as well as the ability of these drugs to penetrate sanctuary sites, including the brain and testis.

The pharmacogenomics of MDR1 must be considered when evaluating novel MDR modulators in patients. The utilization of MDR1 genotyping may reduce some of

the risks associated with clinical trials while at the same time explaining some of the adverse effects, such as neurotoxicity, seen in some patients. This may lead to using genetic parameters to individualize drug therapy and allowing genotype-based dose recommendations to minimize toxicities while maximizing the therapeutic effects.

SNP	Region	Frequency of SNPs (%)		Effect
		Heterozygous	Homozygous Observed	
T-12C	Exon 1	11.8	0	Non-coding
G-1A	Exon 2	11.2	0	Translation initiation
A61G	Exon 2	17.6	0.5	Asn21Asp
G-25T	Intron 4	26.0	3.5	
G-35C	Intron 4	1.2	0	
T307C	Exon 5	1.2	0	Phe103Leu
C+139T	Intron 5	48.2	16.6	
C+145T	Intron 5	2.4	0	
G1199A	Exon 11	12.9	0	Ser400Asn
C1236T	Exon 12	48.9	13.3	Gly412Gly
C+44T	Intron 12	11.7	0	
T-76A	Intron 16	45.9	22.4	
A+137G	Intron 17	1.2	0	
G2677T	Exon 21	43.4	42.2	Ala893Ser
G2995A	Exon 24	11.1	0	Ala999Thr
C3435T	Exon 26	47.7	26.4	Ile1145Ile
C3396T	Exon 26	0.53	0	Wobble

Table 1.2. MDR1 polymorphisms. Adapted from (Brinkmann et al., 2001)

E. Clinical relevance of P-gp and MRP1

The expression of ABC transporters has been extensively studied in a wide variety of cancers to determine what clinical relevance the expression of the transporters has on treatment and prognosis for the patient. It has been observed that MDR affects patients with a variety of cancers, both leukemias and solid tumors, including breast, lung, and brain cancer. Overexpression of P-gp has been documented in a number of tumor types, including leukemias and solid tumors such as breast, lung and brain cancer. This increased expression of P-gp is especially seen after the patient has received chemotherapy, indicating that this mechanism of MDR is clinically important (Bell et al., 1985; Fojo et al., 1987; Ma et al., 1987; Goldstein et al., 1989; Ling, 1997). In contrast, the MRP1 mRNA levels in malignant melanoma (Schadendorf et al., 1995), acute lymphocytic leukemia (Hart et al., 1994) or chronic lymphocytic leukemia (Leveille-Webster and Arias, 1995) were not altered by chemotherapy. Additionally, several studies have shown that P-gp expression can be a prognostic indicator in certain malignancies.

In the case of acute myelogenous leukemia (AML), P-gp expression correlated with reduced rates of complete remission and higher incidence of refractory disease (Leith et al., 1999; van der Kolk et al., 2000). There was no such correlation with either MRP1 or LRP. This is one of the largest trials (352) of untreated patients showing intrinsic resistance (35-43%) and possibly explains why some AML patients respond better to chemotherapy than others (Leith et al., 1999). The expression of P-gp at diagnosis (33%) has been shown to significantly increase to more than 50% in patients that experience relapse with even higher levels in secondary leukemias (Dorr et al., 2001).

Similar conclusions were observed with acute lymphoblastic leukemia (ALL) as well as in AML patients. P-gp overexpression was related to poorer therapy outcome and shorter disease-free survival while MRP1 and LRP overexpression occured so rarely in ALL that it is highly unlikely for its expression to be a prognostic factor (Damiani et al., 2002). Disease-free survival was also significantly higher in ALL patients with initial P-gp negative tumors compared to those with P-gp positive tumors. P-gp expression was more frequently found at relapse than at primary diagnosis (34% vs. 14%). In this relapsed patient group, P-gp positive patients had a two-fold greater risk for adverse clinical outcomes than P-gp negative relapsed patients (Dhooge et al., 1999).

In studies of MRP1 in hematapoietic cells, the levels of expression in normal and malignant cells were equivalent (Abbaszadegan et al., 1994; Schneider et al., 1995). In certain cases such as AML, there have been observations of moderate increases in expression (Hart et al., 1994; Schneider et al., 1995). However, the overexpression of MRP1 is not consistently found in tumors, especially those tumors that are drug-resistant. This inconsistency and low occurrence of expression make any correlation between MRP1 expression and a resistance phenotype difficult in leukemias.

In solid tumors of the breast, clinical observations have shown that 41% of breast tumors express P-gp. These breast cancer patients with P-gp expressing tumors are 3 times more likely to fail chemotherapy than those patients with P-gp negative tumors (Trock et al., 1997). P-gp expression increased after chemotherapy and was associated with a greater likelihood of treatment failure (Mechetner et al., 1998). This correlation can be observed in neuroblastomas where patients with P-gp negative tumors had

significantly longer relapse-free survival and overall survival compared to those patients with P-gp positive tumors (Chan et al., 1991).

Both soft tissue sarcomas and osteosarcomas reveal strong associations between decreased P-gp expression and increased relapse-free and overall survival (Chan et al., 1990; Chan et al., 1997; Coley et al., 2000; Wunder et al., 2000; Gottesman et al., 2002). A rapid upregulation of MDR1 expression can be activated using sarcoma cell lines as well as in human metastatic sarcoma after transient (50 min.) *in vivo* exposure to doxorubicin (Abolhoda et al., 1999). This induction of MDR1 mRNA is also displayed in bladder cancers where a 3.5-5.7 fold increase occurs after receiving systemic chemotherapy in comparison to untreated primary tumors (Tada et al., 2000).

In studies of lung cancer samples, MDR1 mRNA expression was increased in 15-50% of tumors (Oka et al., 1997; Savaraj et al., 1997). For comparison, MRP1 expression was increased in 80% of small-cell lung cancer (SCLC) samples and MRP1 expression was detected in 100% of non-small-cell lung cancer (NSCLC) (Nooter et al., 1995; Young et al., 1999). This is not surprising considering the ubiquitous expression of MRP1 in normal lung tissue. In fact, in some cases, MRP1 expression in lung tumors was found to be lower than those in normal lung tissue (Thomas et al., 1994).

The incidence and expression of MRP1 may possibly contribute to a resistance phenotype, but the number of studies and observations make any conclusion of the role of MRP1 in mediating MDR uncertain. However, the evidence clearly demonstrates a strong correlation between P-gp expression and its mediation of MDR. The evidence suggests that the expression of P-gp and its activity is clinically more significant and relevant than elevation of MRP1 levels.

Overexpression of P-gp has been observed in a variety of cancers, both intrinsically and after the patient has received chemotherapy, indicating the clinical relevance of P-gp-mediated MDR. Furthermore, it has been shown that P-gp expression can be used as a prognostic factor in determining patient response to chemotherapy and overall survival. Treatment of these P-gp expressing tumors with anticancer drug in combination with a chemosensitizing agent that can modulate the activity of P-gp may improve the efficacy of the anticancer drug and reverse the P-gp-mediated MDR phenotype.

F. MDR Modulators

1. First generation modulators

In the past 15 to 20 years, many compounds have been investigated for their ability to reverse MDR in cancer patients. These MDR modulators were initially drugs that were already in use clinically for other indications but were found to also inhibit the transporters, P-gp and MRP1, and reverse the MDR phenotype *in vitro*. The modulators function by blocking the transporter-mediated drug efflux so that a concomitantly administered anticancer drug can cause tumor cell death. These first generation modulators included verapamil (calcium channel blocker) and cyclosporin A (immunosuppressive agent) (Tsuruo et al., 1981; Tan, 2000). Structures of MDR modulators are in Figure 1.1.

There are examples in which these modulators have proven beneficial in the patients. Cyclosporin A, in combination with chemotherapy in retinoblastoma patients, displayed a high cure rate of 91% in previously untreated patients and remained relapse-free over the course of the study (Chan et al., 1996). Addition of cyclosporin A to a chemotherapy regimen consisting of cytarabine and daunorubicin in patients with poor-risk AML significantly reduced resistance to daunorubicin, prolonged the duration of remission and improved overall survival (List et al., 2001). Dose-limiting toxicities were not encountered in these AML patients, but there were altered pharmacokinetics of daunorubicin resulting in increased systemic exposure. While cyclosporin A can also modulate other transporters, including MRP1 and BCRP, the evidence demonstrates the potential of using P-gp modulators in a clinical setting.

A major limitation of the first generation MDR modulators is that they could reverse the MDR phenotype only at high concentrations, which resulted in unfavorable toxicities. Toxicities included cardiac irregularities and myelosuppression (Sikic et al., 1997; Kaye, 1998; Sikic, 1999), which is not surprising considering the initial indications for which the drugs were used. Many of these modulators were substrates for other transporters (i.e. MRP1) and enzyme systems (i.e. P450), which resulted in unpredictable pharmacokinetic interactions with co-administered anticancer drugs (Thomas and Coley, 2003). The toxicities associated with the first generation modulators led to the development of novel compounds that were more potent and selective for P-gp and less toxic.

2. Second generation modulators

The second generation modulators were typically analogs of the first generation modulators including valspodar (PSC 833), dexverapamil, and biricodar (VX-710). These agents are more potent and less toxic than the first generation modulators (Tan, 2000). The most studied and characterized of these agents is the nonimmunosuppressive cyclosporin D analog, valspodar (PSC 833), which has been shown to have a ten-fold increase in potency in reversing MDR activity *in vitro* compared to cyclosporin A (Schuurhuis et al., 1995). *In vivo* studies show that valspodar can reverse resistance to doxorubicin and vincristine in MDR tumor-bearing mice (Watanabe et al., 1995). In phase I trials with valspodar, it was well tolerated in combination with doxorubicin and paclitaxel, but the pharmacokinetic profiles of these drugs were significantly altered by valspodar, which required at least a 60% dose reduction to observe the same degrees of

toxicity (Advani et al., 2001). Phase II trials of valspodar have not been encouraging. In combination with daunorubicin and cytarabine in patients with relapsed and primary refractory acute myeloid leukemia, the modulation of P-gp by valspodar did not significantly improve treatment results (Gruber et al., 2003).

The second generation modulators have a better pharmacological profile than their predecessors but still retain some characteristics that limit their usefulness in the clinic. The major characteristic is that these modulators inhibit the metabolism and excretion of the co-administered anticancer drugs, thereby leading to altered pharmacokinetics and unacceptable toxicities. For example, valspodar is a substrate of cytochrome P450 3A4 and inhibits the metabolism of paclitaxel and vinblastine, which leads to increased concentrations of these cytotoxic drugs and an increased risk of toxicity in patients (Fischer et al., 1998; Bates et al., 2001). This altered pharmacokinetic profile necessitated dose reduction of the anticancer drugs during the clinical trials.

In addition, many of these second generation modulators (i.e. valspodar and biricodar) not only inhibitied P-gp but also several other ABC transporters, including MRP1. It is possible that the inhibition of these non-targeted transporters can lead to increased toxicities and adverse effects associated with the anticancer drugs. This led drug discovery efforts to develop newer modulators that demonstrate improved P-gp selectivity and pharmacological properties.

3. Third generation modulators

Third generation modulators that specifically and potently inhibit P-gp activity have been developed through the use of combinatorial chemistry and structure-activity relationships. These modulators, including XR9576 (tariquidar) (Martin et al., 1999; Stewart et al., 2000), LY335979 (zosuquidar) (Dantzig et al., 1996b), OC-144093 (Newman et al., 2000b; Guns et al., 2002) and R101933 (Gottesman et al., 2002; Thomas and Coley, 2003), have reduced some of the limitations of the second generation modulators (Roe et al., 1999; Lawrence et al., 2001; Smith, 2001). These modulators are not substrates of cytochrome P450 3A4 and do not alter its activity, which can explain why they do not significantly alter the pharmacokinetic profile of co-administered anticancer drugs such as paclitaxel (Dantzig et al., 1996a; Mistry et al., 2001). Also, these modulators usually do not inhibit other ABC transporters such as MRP1, although they have not all been tested against all of the ABC transporters. The specificity for P-gp limits the possibility of the inhibition of another transporter altering the pharmacokinetics or distribution of the anticancer drug. The clinical trials with the third generation modulators have been very promising.

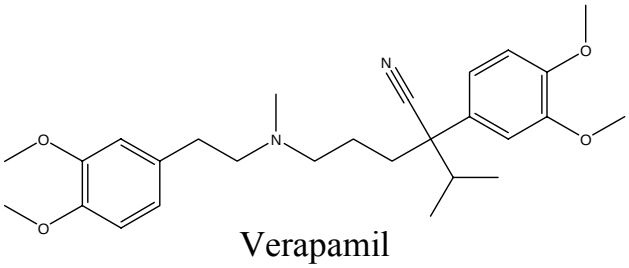
In a phase I trial, XR9576 demonstrated sustained inhibition of P-gp after both intravenous (i.v.) and oral (p.o) administration with an elimination half-life of 24 h (Stewart et al., 2000). XR9576 was well tolerated in patients and showed no alteration in the pharmacokinetic profile of co-administered paclitaxel or doxorubicin. This eliminated the need for dose reduction of the anticancer drugs in these clinical trials. XR9576 is currently in phase III trials with NSCLC patients (Thomas and Coley, 2003).

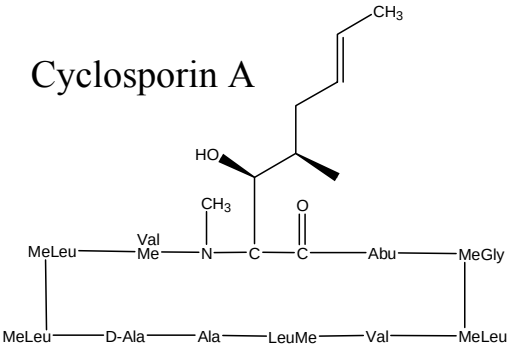
LY335979 (zosuquidar) is a highly selective P-gp inhibitor that does not modulate MRP1 or BCRP-mediated drug resistance (Shepard et al., 2003). In clinical studies in both solid tumors and leukemias, LY335979 did not significantly alter the pharmacokinetics of co-administered doxorubicin, etoposide, or paclitaxel (Dantzig et al., 1996a; Starling et al., 1997).

The third generation modulators have many advantages compared to the previous generations of modulators. These modulators are more specific and potent inhibitors of P-gp, generally lacking interaction with cytochrome P450 3A4, and usually allowing for full therapeutic doses of co-administered cytotoxic drugs in clinical trials due to minimal alteration of the pharmacokinetics. However, these modulators are not without their disadvantages. In a Phase I study with LY335979 (zosuquidar.3HCl trihydrochloride) administered orally in combination with doxorubicin, neurotoxicity was a dose-limiting toxicity characterized by cerebellar dysfunction, hallucinations, and palinopsia (Rubin et al., 2002). Further refinement of the doses and dose scheduling is necessary to determine if P-gp inhibition can be achieved without cerebellar toxicity. In a phase I study with R101933 administered i.v. with docetaxel, patients with advanced malignancies experienced toxicities including neurotoxicity, neutropenic fever, fatigue and myalgia (van Zuylen et al., 2002). Although the current third generation modulators have shown promising results in limited clinical trials, there are still significant toxicities associated with their use in cancer patients. Therefore, this necessitates the development and characterization of novel P-gp-specific MDR modulators with limited toxicities. The ultimate success of these modulators will be determined in ongoing clinical trials.

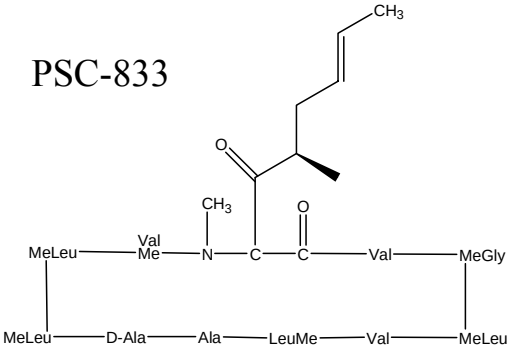
In the future, these modulators may be used not only for chemotherapy but also for chemoprevention (Gottesman et. al., 2002). The previous clinical trials have focused on reversing existing drug resistance through the modulation of P-gp. However, another aim could be the prevention of acquired drug resistance by tumors (strategies to block the upregulation of P-gp). Typically, selection of drug resistant cells *in vitro* begins with exposure to low concentrations of drug and gradually increasing concentrations over time. High concentrations of drugs initially would markedly decrease the number of resistant clones isolated. Therefore, a strategy to prevent drug resistance would be to increase the intracellular concentration of anticancer drugs by administering a modulator at the beginning of treatment, instead of after drug resistance has been established, to limit the number of resistant clones that may emerge. In a clinical trial of modulators with the aim of preventing drug resistance, significant differences in patient response rates may not be seen; only differences in rate of relapse and time of progression. This is because a chemoprevention strategy with the modulators would only target a select population of tumor cells without affecting a majority of the cells and thereby not signicantly alter initial cytotoxicity. In a single-step selection, it has been shown *in vitro* that co-administration of a modulator reduces the rate of mutations that cause doxorubicin resistance while suppressing the expression of P-gp, and in clinical trials, the initial combination treatment of AML patients with cyclosporin A and daunorubicin/Ara-C improved overall survival and disease-free survival although with no impact on the complete remission rate (Beketic-Oreskovic et al., 1995; List et al., 2001). The area of chemoprevention with P-gp specific MDR modulators is still in its infancy but may lead to a novel approach of eliminating the acquisition of the MDR phenotype by tumors.

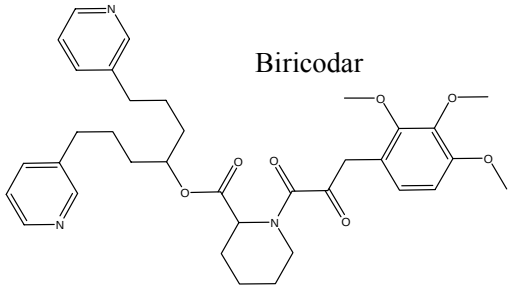
First generation modulators



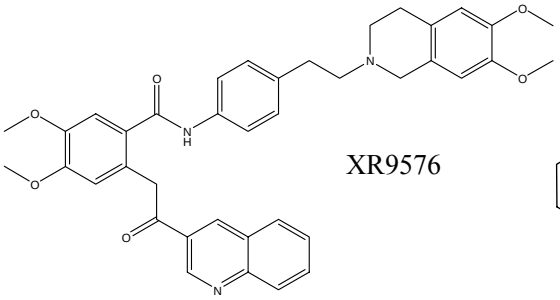


Second generation modulators





Third generation modulators



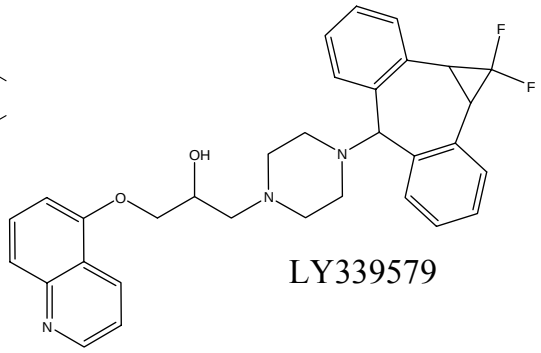


Fig. 1.1. Structures of modulators.

G. Discovery and development of P-gp antagonists

Due to the need for improved P-gp-specific MDR modulators, the development of novel inhibitors continues. In the discovery and development of new P-gp inhibitors, there are a number of steps that are involved, including (1) target selection and validation, (2) assay development, (3) screening, (4) compounds for drug discovery, (5) lead optimization, (6) preclinical *in vivo* evaluation, and (7) clinical trials.

1. Target selection and validation

Target selection and validation involves the identification of new targets that are relevant and specific to a particular disease and may be manipulated by drugs. The majority of drug targets include receptors, enzymes and ion channels. The biochemical and molecular analyses of P-glycoprotein has been extensively characterized over the past 25 years. In addition, the expression of P-gp and its relevance to clinical oncology and drug-resistance has been well established. Therefore, the target selection of P-glycoprotein for the treatment of multidrug resistant cancers has been well validated. The selection and validation of a target is extremely important because if the target is not relevant or specific for a particular disease, even the most specific inhibitor will not affect the disease state.

2. Assay development and screening

Assay development and screening are critical to finding potential substrates or inhibitors of P-gp. A number of factors must be considered when developing screening assays, including relevance, selectivity, sensitivity and reproducibility, as well as speed and cost. Relevance refers to the fact that the assay should mimic the desired *in vivo* activity (i.e. P-gp overexpressing cell lines becoming more drug-sensitive to cytotoxic P-

gp substrates through the inhibition of activity). Selectivity of an assay is crucial because if the desired effect is seen in the assay, it may not be from inhibition or modulation of the target if the assay is not selective enough. Sensitivity of the assay allows for the identification of low-affinity compounds, especially when screening mixtures of compounds such as from a crude extract of natural products. All assays have variability, but reproducibility is necessary in screening since compounds are usually only tested one time at a single dose. Minimizing variation in the assay will lead to a reduced risk of missing positive hits or of chasing false positives. As with any assay, speed and cost are key factors, especially considering the number of compounds that are usually screened in any given assay. In the case of assays for screening P-gp inhibitors, there are a number of assays available that have led to numerous positive hits that have been further developed and characterized. These assays include indirect fluorescence indicator dye assays where a fluorescent dye (i.e. rhodamine 123 or calcein-AM), which is a P-gp substrate, is used to determine the effect of other compounds in inhibiting the efflux of these dyes from P-gp expressing cells (Schwab et al., 2003). *In vitro* cytotoxicity assays using a colormetric substrate (i.e sulforhodamine B) can also be used with P-gp-expressing cells to determine if compounds can potentiate the cytotoxicity of drugs that are substrates for P-gp and reverse the drug-resistant phenotype displayed in these cells (Skehan et al., 1990). These assays are suitable for high throughput screening and have generated a number of positive hits for further development of P-gp inhibitors.

3. Compounds for drug discovery

Compound collections for drug discovery are divided into two major classes: natural products and synthetic compounds. The advantages of screening for inhibitors of

P-gp using natural products are their chemical diversity and evolutionary optimization, where specificity has been refined. The major disadvantage of natural products is their chemical complexity, which can make their synthesis difficult and can result in biological instability. This difficulty in synthesis of natural products has made synthetic compounds more attractive since the compounds can more easily be produced in larger amounts for further development. However, synthetic compounds have a disadvantage in that the chemical diversity can be quite limited. There are ways in which the limited diversity of synthetic compound libraries can be used to create compounds that are likely to have drug-like properties, assuming that novel inhibitors will share similar chemical properties with current drugs available (Xu and Stevenson, 2000).

The screening of compounds for inhibitors of P-gp or drugs that can reverse the P-gp-mediated MDR phenotype has resulted in a wide range of compounds with different pharmacological properties (Table 1.3). These P-gp modulators can be divided into three functional categories: (1) high-affinity substrates of P-gp, (2) inhibitors of ATP hydrolysis coupled P-gp transport, and (3) partial substrate inhibitors (Wang et al., 2003).

Since the discovery of the ability of verapamil (first generation modulator) to competitively inhibit P-gp activity, there has been much research devoted to the development of inhibitors that can effectively reverse the MDR phenotype (Tsuruo et al., 1981). Azidopine has been extensively used as a photoaffinity substrate of P-gp to determine the competitive inhibition of binding of other modulators (Safa et al., 1987). Quinidine was once considered a very promising MDR-reversing agent, but a phase III clinical trial resulted in its ineffectiveness as an MDR modulator of epirubicin in the treatment of metastatic breast cancer (Fisher and Sikic, 1995).

Trifluoperazine, a calmodulin antagonist, was identified as a chemosensitizer of P-gp (Ford et al., 1989; Ford, 1996). Subsequent development of calmodulin antagonists and structural analogs resulted in more potent P-gp inhibitors such as LY335979, R101933, and GF120918. GF120918 has been shown to be a potent inhibitor of P-gp and BCRP and can increase the oral bioavailability of paclitaxel 7-fold in comparison to cyclosporin A (Malingre et al., 2001; Edwards et al., 2002).

P-gp is a well known substrate for phosphorylation by several protein kinases including protein kinase A (Mellado, 6900, 1987) and protein kinase C (PKC) (Chambers, 309, 1994)(Chambers, 4592, 1993). The phosphorylation of P-gp by PKC results in the alteration of function and binding ability of P-gp (Ahmad, 10313, 1994). Therefore, the inhibition of PKC was thought to prevent drug transport and reverse MDR phenotype. The PKC inhibitors, including staurosporine, inhibited the activity of P-gp but displayed high intrinsic cytotoxicity in both parasite cell lines and human MDR cells (Smith and Zilfou, 1995).

The speculation of a physiological role of P-gp in the transport of steroids led to the development of steroids and its structural analogs as P-gp inhibitors. Progesterone has been shown to bind to P-gp without being transported by it (Ueda et al., 1992). Tamoxifen appears to reverse MDR through a similar mechanism as progesterone, but in clinical studies, it was poorly tolerated at doses necessary to achieve serum concentrations for effective P-gp inhibition (Stuart et al., 1992; Callaghan and Higgins, 1995).

The cyclic peptides cyclosporin A and FK-506 are important immunosuppressants in organ transplantation, but can also act as P-gp modulators (Lo and Burckart, 1999).

Cyclosporin A has been extensively studied as a MDR modulator with inhibition of P-gp seen in *in vivo* models. However, cyclosporin A also alters the pharmacokinetics and pharmacodynamics of co-adminstered drugs and shows signs of systemic toxicity. PSC-833, a non-immunosuppressive analog of cyclosporine, was shown to be 10-to 30-fold more potent *in vitro* than cyclosporin A (Boesch et al., 1991). However, clinical trials with PSC-833 have not been promising.

The different categories of drugs that can effectively inhibit P-gp cover a diverse set of compounds. Since P-gp can be inhibited by so many different compounds, it necessitates that the libraries of compounds used for screening must be chemically diverse as well. Only through the use of structurally-diverse libraries can novel P-gp inhibitors be discovered and further developed.

Category	Name	Active Concentration (μM)
Calcium and sodium channel blockers	Verapamil	7.5
	Azidopine	0.76
	Quinidine	10
	Primaquine	210
	Amiodarone	4
	Propafenone	2.3
Calmodulin antagonists and structural analogs	Trifluoperazine	4
	LY335979	0.06
	GF120918	0.06
	R101933	0.51
Protein kinase C inhibitors	Dexniguldipine	2.5
	Staurosporine	5.0
	Calphostin C	0.25
Steroids and structural analogs	Progesterone	2
	Tamoxifen	1.35
	RU486	0.53
Indole alkaloids, cyclic peptides and macrolide compounds	Yohimbine	250
	Cyclosporin A	1.4
	PSC-833	1.13
	FK-506	0.74
	Rapamycin	3
Others	XR9576	0.02
	Biricodar	1.25
	OC144-093	0.09
	Itraconazole	1.7
	Ketoconazole	6
	Dipyridamole	7.5

Table 1.3. P-glycorprotein inhibitors. Adapted from (Wang, 203, 2003)

4. Lead optimization

Once a compound or family of compounds has been shown to have activity, it becomes a lead structure. At this point the objective is to enhance the activity, selectivity and bioavailability of the compound for the target, P-gp. There are a number of methods for lead optimization, including modeling the enzyme substrates and substituting bioisosteric groups or using quantitative structure-activity relationships (QSAR). QSAR attempts to quantitatively relate biological activity to chemical properties through the use of a mathematical formula. The higher quality and more relevant the biological data is, the more predictive of activity the formula will be. Recent studies of the structure-activity relationship of P-gp inhibitors and substrates indicate that MDR reversal activity correlates to the lipophilicity, molecular weight, longest chain of the molecule and the energy of the highest occupied orbital (Wang et al., 2003). QSAR analyses of P-gp inhibitors can be useful in the development of new P-gp-specific MDR modulators by determining what chemical parameters an effective inhibitor should possess.

5. *In vivo* models

The compounds developed must be tested in an *in vivo* model. Many of the compounds discussed earlier displayed excellent reversal of MDR *in vitro*, but failed to achieve clinical success for a number of reasons, including the intrinsic toxicity at the high doses required to achieve the serum concentrations necessary to inhibit the transporters and the alteration of the pharmacokinetics and pharmacodynamics of the co-administered anticancer drugs (Sikic et al., 1997). A necessity of an *in vivo* model is its predictive value of whether a drug will be successful in a clinical trial. A significant limitation in the drug development effort has been the lack of predictive *in vivo* tumor

models for the evaluation of MDR modulators. Models commonly used include the intraperitoneal growth of P388 mouse leukemia cells and their drug-resistant, P-gp overexpressing derivative, P388/ADR (Johnson et al., 1978; Tsuruo et al., 1981; Dantzig et al., 1996b). There are some advantages in utilizing this model, including low expense and good reproducibility. However, the use of a non-epithelial cancer cell line and the administration of both the modulator and cytotoxic drug directly into the intraperitoneal cavity in which the cells have been implanted produce a sub-optimal system since systemic distribution of drug and modulator are not required. Other models involve xenograft transplantation of resistant human epithelial tumor cells to form solid tumors in immunocompromised animals (Plumb et al., 1994; Mistry et al., 1999). The greatest advantage of this model is the use of human tumor cells that express human P-gp, which allows for the modulation of human P-gp as compared to mouse P-gp expressed in the previously described P388/ADR model. Also, the results of experiments with xenograft models have been shown to be more relevant to clinical practice than those obtained from the P388 ascites model (Boven et al., 1992). Other advantages include the opportunity to delay treatment until the formation of a palpable tumor, as well as the accessibility of the external tumors for serial measurements of volume. Disadvantages of this model include the expense of immunocompromised mice and the inability to examine the effect of the immune system on the tumor, as well as any toxicity of the drug and modulator toward the immune system. Another model that has been used involves the transplantation of the Colon26/MC26 murine tumor cell line (Spoelstra et al., 1991; Watanabe et al., 1995). However, in this model, treatment usually begins 24-72 h after implantation, which does not allow for the solid tumor to adhere and proliferate, which contrasts with the clinical

setting in which treatment typically begins after tumor growth is quite advanced. Also, this model does not allow for tumor adherence, growth and palpability (Spoelstra et al., 1991; Watanabe et al., 1995). While these models allow the evaluation of certain parameters of the test modulator, their disadvantages limit their overall utility. Therefore, there is a need for the development of *in vivo* tumor models that incorporate many of the advantages of the previously mentioned models while limiting their disadvantages.

6. Clinical trials

Clinical trials consist of three main phases. Phase I is to establish the maximum tolerated dose in humans and to examine the pharmacokinetics in a small number of patients (20-50). Efficacy is not the focus, but the responses to the drug are noted. Phase II is to determine the efficacy of the drug and continue the monitoring of toxicities. This phase consists of 50-300 patients with carefully matched disease. Phase III studies are the largest studies (greater than 1000 patients) where the efficacy and safety of the drug is verified. The large number of patients is required to ensure that no inconsistent symptoms (i.e spontaneous cancer remission) account for the actual efficacy of the drug.

The clinical trials of P-gp inhibitors were not very successful with the early generation modulators. This was due to toxicities and unpredictable pharmacokinetics of the co-administered drugs. However, these problems may also arise in the later generation modulators due to MDR1 polymorphisms. As previously described, there are genetic variations within the MDR1 gene that influence the function or expression of P-gp. This can alter the pharmacokinetics and pharmacodynamics and impact the therapeutic efficacy of the inhibitors. The pharmacogenomics of MDR1 must be considered when evaluating novel MDR modulators in clinical trials.

Another concern with the clinical trials of P-gp inhibitors is that the patient population tested is generally not analyzed for P-gp expression. Therefore, the differences seen between patients may be due to changes in the P-gp expression of the tumors. Monitoring of P-gp expression should occur before the clinical trial to ensure that the patient population expresses the drug target to which efficacy of the drug is being tested against. Patients with MDR tumors that are not mediated through P-gp expression will not show good clinical efficacy of P-gp inhibitors. The success of P-gp-specific MDR modulators in ongoing clinical trials will determine whether the inhibition of P-gp in combination with anticancer drugs can reverse the MDR phenotype and lead to greater survival for cancer patients.

H. Conclusion

The clinical significance of P-gp as well as its less ubiquitous tissue expression as compared to MRP1 makes P-gp a more suitable drug target to reverse MDR. It is likely that the lack of ABC transporter-selectivity as well as their alterations in drug metabolism and excretion played a role in the failure of the early generation MDR modulators. The preliminary results of the third generation P-gp specific MDR modulators present a brighter future in realizing the goal of P-gp inhibition, effectively reversing drug resistance in humans and leading to increased overall survival and a better quality of life. However, there is a continuing need for the elucidation and development of new P-gp inhibitors that maximize the therapeutic effect while minimizing the toxicities.

CHAPTER 2

HYPOTHESES

Multidrug resistance (MDR) is a phenomenon in which tumors are either intrinsically resistant or acquire a resistance to cytotoxic drugs that are normally used in chemotherapy regimens. One primary mechanism by which tumors achieve MDR is through the expression of P-glycoprotein (P-gp), a drug efflux pump located in the plasma membrane. P-gp functions by removing cytotoxic drugs from inside cells, thereby protecting tumors from these drugs. Therefore, modulators that specifically inhibit P-gp can be co-administered with cytotoxic drugs to increase their efficacy and prevent their efflux by P-gp. When given at doses necessary to inhibit P-gp, the early generation modulators tested in clinical trials exhibited toxicities and altered pharmacokinetics of the concomitantly administered drugs. The failure of these modulators may have been due to lack of selectivity, which can lead to the modulation of other enzymes such as MRP1 and cytochrome P450. This can result in increased drug distribution in normal tissues as well as decreased metabolism of the cytotoxic drugs. We believe that the selectivity of these antagonists to P-gp is critical to their utility as clinical agents. The focus of this project is to develop MDR modulators that specifically inhibit P-gp, increase the efficacy and potency of co-administered cytotoxic drugs and show efficacy in an *in vivo* model. The development of compounds that can reverse drug

resistance or maintain cytotoxicity towards MDR tumors would be a significant improvement to current chemotherapy protocols.

Establishment of *in vivo* tumor models. There are a number of *in vivo* tumor models used to evaluate the efficacy of MDR modulators. Most of the first generation MDR modulators displayed good efficacy in these models. However, these results did not translate to success in clinical trials. The lack of predictive *in vivo* tumor models for the evaluation of MDR modulators has been a significant limitation in the drug development efforts of these modulators. Therefore, we hypothesize that an *in vivo* solid tumor model with a spontaneous transformed cell line using immunocompetent animals would more closely resemble the environment in which human tumors are treated in a clinical setting and may be a more predictive model for clinical success.

Characterization of P-gp-specific MDR modulators. It is likely that the lack of transporter-selectivity in early generation MDR modulators played a role in their ultimate failure. Therefore, we hypothesize that selectivity of individual MDR modulators to antagonize P-gp and increase potency will lead to less systemic toxicity and decreased alteration of the pharmacokinetics of the co-administered cytotoxic drugs. To identify P-gp selective inhibitors, we screened a library of drug-like molecules and discovered a number of pharmacophores. The studies herein describe the *in vitro* and *in vivo* characterization of these P-gp selective modulators.

Cellular influx mechanism of actinomycin D transport. The development and characterization of P-gp-specific MDR modulators has led to an interesting observation between 2-benzyloxy-3-methyl-quinoxaline (BMQ) and the antitumor drug, actinomycin D. Compound BMQ is able to increase the intracellular accumulation of actinomycin D

in a variety of cell lines. This facilitation of accumulation of actinomycin D by BMQ did not appear to be a passive diffusional process. Therefore, we hypothesize that actinomycin D enters the cell through an active transport process, and we seek to analyze properties of carrier-mediated transport including saturability, temperature-dependence and energy-dependence.

CHAPTER 3

DEVELOPMENT OF A SYNGENEIC *IN VIVO* TUMOR MODEL FOR THE CHARACTERIZATION OF P-GP-SPECIFIC MULTIDRUG RESISTANCE MODULATORS

A. Introduction

A major deterrent to successful chemotherapy of cancer is the phenomenon of MDR, in which tumors demonstrate an intrinsic or acquired resistance to a variety of structurally unrelated anticancer drugs. One major mechanism of MDR is through the overexpression of the energy-dependent, unidirectional transmembrane efflux pump, P-gp. P-gp, a 170 kDa protein, belongs to the ATP-binding cassette (ABC) superfamily of transporters (Juliano and Ling, 1976; Taylor et al., 2001). P-gp functions by binding to anticancer drugs within the cell and releasing them to the extracellular space using energy from the hydrolysis of ATP (Horio et al., 1988). Tumor cells that are exposed to cytotoxic compounds often overexpress P-gp, thereby allowing these cells to survive treatment with anticancer drugs by reducing their intracellular accumulation. Efflux of the anticancer drugs blocks their efficacy by preventing their interaction with intracellular targets (Kartner et al., 1983).

Overexpression of P-gp has been documented in a number of tumor types including acute leukemia and small-cell lung carcinoma, especially after the patient has received chemotherapy (Ling, 1997). Additionally, several studies have shown that P-gp expression can be a prognostic indicator in certain malignancies. For example, increased expression of P-gp in neuroblastoma and childhood sarcoma is associated with poor response to chemotherapy and decreased survival (van de Vrie et al., 1998). Furthermore, breast cancer patients with P-gp expressing tumors are three times more likely to fail chemotherapy than those patients with P-gp negative tumors (Trock et al., 1997).

Because of the importance of P-gp in determining the success of chemotherapy, attempts have been made to develop compounds that act as antagonists of P-gp. These antagonists, often termed MDR modulators, generally lack anticancer activity by themselves. Their function is to block P-gp-mediated drug efflux so that a concomitantly administered anticancer drug can cause tumor cell death. Some of the initial attempts to develop MDR modulators focused on the drugs verapamil and cyclosporin A (first generation modulators) (Tan, 2000). These compounds demonstrated excellent reversal of MDR *in vitro*, but failed to achieve clinical success for several reasons, including: their intrinsic toxicity at the high doses required to achieve the serum concentrations necessary to inhibit the transporters; their alteration of the pharmacokinetics of the co-administered anticancer drugs; and their metabolism by cytochrome P450s (Sikic et al., 1997). More recent MDR modulators (i.e. LY335979, XR9576, substituted quinoxalinones and azabicyclooctanes) tend to be more potent than the early modulators, and in some cases show greater selectivity for P-gp (Hyafil et al., 1993; Dantzig et al., 1996b; Newman et al., 2000a; Lawrence et al., 2001; Smith, 2001).

A significant limitation in these drug development efforts has been the lack of predictive *in vivo* tumor models for the evaluation of MDR modulators. A commonly used *in vivo* model involves the intraperitoneal growth of P388 mouse leukemia cells and their drug-resistant, P-gp overexpressing derivative, P388/ADR (Johnson et al., 1978; Tsuruo et al., 1981; Dantzig et al., 1996b). However, the use of a non-epithelial cancer cell line, and the administration of both the modulator and cytotoxic drug directly into the cavity in which the cells have been implanted produce a sub-optimal system since systemic distribution of drug and modulator are not required. Other models involve xenograft transplantation of resistant human epithelial tumor cells to form solid tumors in immunocompromised animals (Plumb et al., 1994; Mistry et al., 1999). Disadvantages of this model include the expense of immunocompromised mice and the inability to examine the effect of the immune system on the tumor, as well as any toxicity of the drug and modulator toward the immune system. Another model that has been used involves the transplantation of the Colon26/MC26 murine tumor cell line (Spoelstra et al., 1991; Watanabe et al., 1995). However, in this model, treatment usually begins 24-72 h after implantation, which does not allow for the solid tumor to adhere and proliferate, which contrasts with the clinical setting in which treatment typically begins after tumor growth is quite advanced. While these models allow for the evaluation of certain parameters of the test modulator, their disadvantages limit their overall utility. Therefore, we have developed an *in vivo* tumor model using immune-competent mice that incorporates many of the advantages of the previously mentioned models while limiting their disadvantages.

B. Materials and Methods

Cell culture and cell lines. Cell lines CRL-2116 (ATCC designation: JC), TIB-75 (BNL 1ME A.7R.1), TIB-76 (BNL 1NG A.2), and CRL-1638 (NMuLi) were obtained from the American Type Culture Collection (Manassas, VA) and cultured as recommended. MCF-7 and NCI/ADR cells were obtained from the Division of Cancer Treatment of the National Cancer Institute. MCF-7/VP cells were provided by Drs. Schneider and Cowan. MCF-7, NCI/ADR, MCF-7/VP, and JC cells were maintained in RPMI 1640 (Life Technologies, Inc., Rockville, MD) with L-glutamine containing 10% FBS and 50 µg/ml gentamicin. TIB-75, TIB-76, and NMuLi cells were maintained in DMEM containing 10% FBS, 1mM sodium pyruvate and 50 µg/ml gentamicin. Cells were maintained at 37°C and 5% CO_2 in 100 mm x 20 polystyrene tissue culture dishes.

***In vitro* cytotoxicity assay.** Cells were seeded in 96-well tissue culture dishes at approximately 20% confluency and allowed to recover and attach for 24 h. Cells were then treated with varying concentrations of modulators and/or cytotoxic drugs for 48 h. The number of surviving cells remaining in each well was quantitated with the sulforhodamine B (SRB) colorimetric assay (Skehan et al., 1990). Briefly, cells were washed with phosphate-buffered saline (PBS) and fixed to the plate with 10% trichloroacetic acid. The cells were then washed with water and stained with 0.4% SRB in 1% acetic acid. Cells were then rinsed with 1% acetic acid and 10 mM Tris base buffer was added to dissolve the SRB. The degree of absorbance was determined with a PerkinElmer HTS 7000 Plus BioAssay plate reader at a wavelength of 570 nm.

***In vitro* drug accumulation assay.** Cells were seeded in 24-well tissue culture dishes at approximately 25% confluency and allowed to recover and grow to near confluency, approximately 3-4 days. Media was aspirated and replaced with serum-free media. Modulators were incubated for 30 min. at 37°C. Approximately 0.1 μCi of [^{3}H]Taxol, 75 Ci/mmol, or [^{3}H]vinblastine, 7.3 Ci/mmol, (Moravek Biochemicals, Brea, CA) was then added per well and the cultures were incubated for 60 min. at 37°C. Radioactive media was aspirated and cells were rapidly washed twice with ice-cold PBS. Intracellular [^{3}H]drug was solubilized with 1% sodium dodecyl sulfate (SDS) in water and quantified by liquid scintillation counting using UniverSol (ICN, Costa Mesa, CA) (Smith et al., 1995).

Western blot analysis. Membrane samples were prepared from MCF-7, NCI/ADR, MCF-7/VP, JC, TIB-75, TIB-76 and NMuLi cells (Smith et al., 1995). Briefly, cells were washed with PBS, detached by gentle scraping and collected by centrifugation at 500 x g for 5 min. The cells were incubated with 10 mM KCl, 1.5 mM $MgCl_2$, 5 μM phenylmethylsulfonyl fluoride, and 10 mM HEPES, pH 7.4, on ice for 30 min. Cells were lysed by gentle homogenization and the lysate was centrifuged at 500 x g for 10 min to remove nuclei and debris. The supernatant was centrifuged at 100,000 x g for 60 min at 4°C and the resulting membrane pellet was resuspended in lysis buffer and stored at -75°C until use. Protein concentrations were determined using a fluorescamine protein assay (Miedel et al., 1989). Equal amounts of protein were loaded for each sample (typically 20 μg). Samples were run on a 7.5% SDS-polyacrylamide gel, and proteins were transferred to a PVDF membrane overnight at 50 V. The membrane was blocked with 5% non-fat dry milk then incubated with 1:100 dilution of mdr1 polyclonal rabbit

antibody (Oncogene Research Products, Boston, MA) or 1:20 dilution of MRP1r1 monoclonal rat antibody (Alexis Biochemicals, San Diego, CA) for 60 min, followed by three 10 min washings, and incubation with a 1:16,000 dilution of the secondary antibody, mouse monoclonal anti-rabbit IgG perioxidase conjugated, or a 1:40,000 dilution of anti-rat IgG perioxidase conjugated (Sigma-Aldrich, St. Louis, MO) for 60 min. Immunoreactive proteins were subsequently visualized using the Western Lightning Chemiluminescence Reagent Plus kit (PerkinElmer Life Sciences, Boston, MA) and exposing the membrane to Kodak Scientific Imaging film (Eastman Kodak Co., Rochester, NY).

***In vivo* growth and treatment.** Animal care and procedures were in accordance with guidelines and regulations of the Institutional Animal Care and Use Committee of The Penn State College of Medicine. Balb/c female mice (Charles River Laboratories, Inc.), 6-8 weeks old, were housed (5 per cage) under 12 h light/dark cycles with food and water provided *ad libitum*. The animals were injected in the subcutaneous space of the right hind quarter with 10^6 JC cells suspended in PBS. After palpable tumor growth, approximately 2-3 weeks after injection, tumor volume was determined (day 1) using calipers measuring the length (L) and width (W) of the tumor. Tumor volume was calculated using the equation: $(L \times W^2)/2$, and animals were randomized into four groups (n=5 per group). Treatment was then administered on days 1, 5, and 9, and consisted of either an intravenous administration of a known P-gp modulator, cyclosporine injection USP (Bedford Laboratories, Bedford, OH) at a dose of 50 mg/kg, with or without the i.v. administration of the cytotoxic drug, doxorubicin hydrochloride (Sigma-Aldrich Co., St. Louis, MO) at a dose of 5 mg/kg. Tumor volumes were monitored until day 15 when the

animals were euthanized (Watanabe et al., 1995). Unpaired t-test with Welch correction was performed using GraphPad InStat version 3.01 for Windows 95 (GraphPad Software, San Diego, CA).

C. Results

1. JC and TIB-75 cells show the P-gp mediated MDR phenotype *in vitro*.

To determine which of four transformed murine cell lines exhibited the classical P-gp-mediated MDR phenotype, cytotoxicity assays were performed using a known P-gp modulator, verapamil, and a cytotoxic drug, paclitaxel, which is a substrate of P-gp. If P-gp is active in the cells, the addition of the modulator should increase the cytotoxicity of the concomitantly administered cytotoxic drug. As shown in Figures 3.1A and 3.1B, the JC and TIB-75 cells were less sensitive to paclitaxel in the presence of the vehicle control, ethanol, than when treated with 20 μM verapamil, indicating potentiation of the cytotoxicity of paclitaxel by verapamil. The IC_{50} for paclitaxel in the JC cells shifted from 219 ± 55 nM (n=6) to 6.5 ± 3.9 nM (n=6) for cells treated with verapamil. Similarly, the IC_{50} for paclitaxel in the TIB-75 cells shifted from 120 ± 45 nM (n=5) to 6.2 ± 2.2 nM (n=5) in the presence of verapamil. These results are consistent with P-gp-mediated MDR in these two cell lines. In contrast, two other transformed murine cell lines, TIB-76 and NMuLi, did not show a P-gp-mediated MDR phenotype. The addition of verapamil did not decrease the concentration of paclitaxel necessary to decrease cell survival by 50% (Figures 3.1C and D). Verapamil, as well as the known MDR

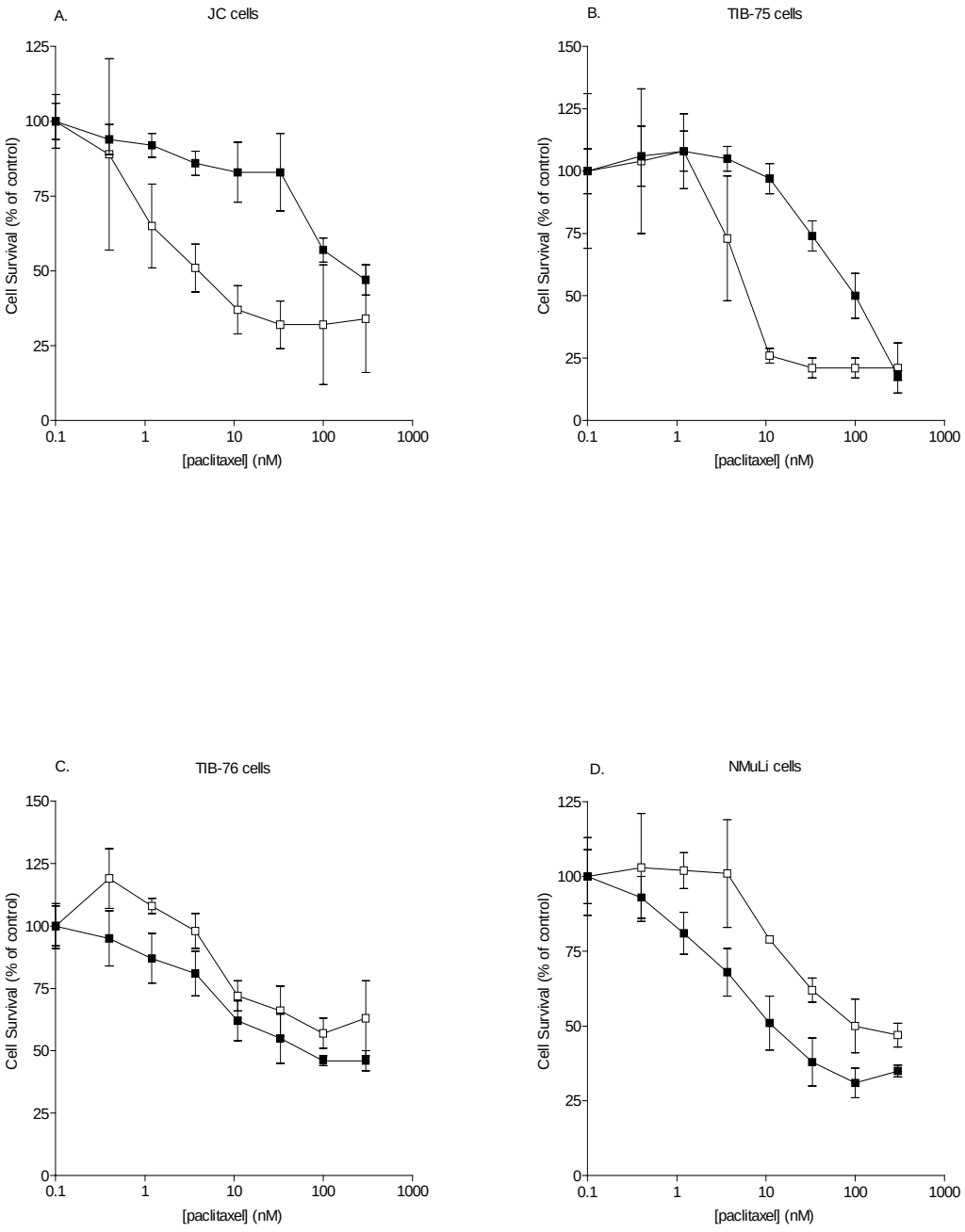


Figure 3.1. *In vitro* cytotoxicity assays with murine cell lines. Four murine cell lines: (A) JC, (B) TIB-75, (C) TIB-76, and (D) NMuLi, were treated with varying concentrations of paclitaxel in the presence of ethanol (■) or 20 μM verapamil (□) to determine their P-gp-mediated MDR phenotype as described in "Materials and Methods". The percentage of cell survival was calculated by comparing drug-treated cells to control cells treated with equivalent amounts of vehicle (ethanol). Results shown as mean ± SE for triplicate cultures.

modulators cyclosporin A and probenicid, were also tested for their abilities to alter the cytotoxicities of several anticancer drugs toward the four murine cell lines (Table 3.1).

These results demonstrated that verapamil causes a similar shift in the IC_{50}s for vincristine, vinblastine and doxorubicin for the JC and TIB-75 cell lines, consistent with the known substrate-specificity of P-gp. Cyclosporin A also sensitized the JC and TIB-75 cells to the P-gp substrate drugs. The reversal indices for these two cell lines were significantly higher than those for the other two cell lines, TIB-76 and NMuLi, which did not exhibit any significant change in the IC_{50}s in response to verapamil or cyclosporin A. Of note, the MRP1-specific modulator, probenecid, did not have any effect on the IC_{50} for vincristine, an MRP1 substrate, in the JC or TIB-75 cell lines. The cytotoxic drug, cisplatin, was used as a negative control since it is neither a substrate of P-gp or MRP1. None of the cell lines were sensitized to cisplatin by either verapamil or cyclosporin A. The NCI/ADR (overexpresses P-gp), MCF-7/VP (overexpresses MRP1), and MCF-7 (non-resistant) cell lines were used for comparison. As expected, the NCI/ADR cells demonstrated large reversal indices to P-gp substrate drugs when treated with verapamil. Reversal of MRP1-mediated resistance was demonstrated by the sensitization of the MCF-7/VP cells to vincristine by probenecid.

Another well-established method for determining P-gp activity is the use of intracellular drug accumulation assays, e.g. with [^{3}H]vinblastine that is normally effluxed from the cell by P-gp. When the cells are treated with a P-gp modulator, there is an increase in the intracellular accumulation of the [^{3}H]vinblastine. As shown in Figure 3.2, the JC and TIB-75 cells displayed significant increases in the intracellular accumulation of [^{3}H]vinblastine as the concentration of verapamil increased. Maximal effects were

Table 3.1 In vitro cytotoxicity and reversal with modulators

Cell Line	Cytotoxic Drug	Modulator	IC50 (nM)	Reversal Index	Cell Line	Cytotoxic Drug	Modulator	IC50 (nM)	Reversal Index
JC	Vincristine	None	10	--	**TIB-75**	Vincristine	None	15	--
		Verapamil-10 µM	0.6	16.7			Verapamil-10 µM	0.5	30.0
		Probenecid-100 µM	10	1.0			Probenecid-100 µM	15	1.0
	Vinblastine	None	5	--		Vinblastine	None	3	--
		Verapamil-10 µM	0.2	25.0			Verapamil-10 µM	0.1	30.0
	Paclitaxel	None	150	--		Paclitaxel	None	100	--
		Verapamil-10 µM	6	25.0			Verapamil-10 µM	5	20.0
	Doxorubicin	None	400	--		Doxorubicin	None	30	--
		Cyclosporin A-4 µM	100	4.0			Cyclosporin A-4 µM	15	2.0
		Verapamil-20 µM	100	4.0			Verapamil-20 µM	10	3.0
	Cisplatin	None	7000	--		Cisplatin	None	1900	--
		Cyclosporin A-4 µM	10000	0.7			Cyclosporin A-4 µM	1000	1.9
		Verapamil-20 µM	7000	1.0			Verapamil-20 µM	2000	1.0
TIB-76	Vincristine	None	0.2	--	**NMuLi**	Vincristine	None	1	--
		Verapamil-10 µM	0.2	1.0			Verapamil-10 µM	1	1.0
	Vinblastine	None	1.2	--		Vinblastine	None	1	--
		Verapamil-10 µM	1.2	1.0			Verapamil-10 µM	1	1.0
	Paclitaxel	None	40	--		Paclitaxel	None	10	--
		Verapamil-10 µM	100	0.4			Verapamil-10 µM	10	1.0
	Doxorubicin	None	150	--		Doxorubicin	None	150	--
		Cyclosporin A-4 µM	150	1.0			Cyclosporin A-4 µM	150	1.0
		Verapamil-20 µM	150	1.0			Verapamil-20 µM	150	1.0
	Cisplatin	None	6000	--		Cisplatin	None	5000	--
		Cyclosporin A-4 µM	5600	1.1			Cyclosporin A-4 µM	7000	0.7
		Verapamil-20 µM	10000	0.6			Verapamil-20 µM	7000	0.7
MCF-7	Vinblastine	None	1.2	--	**NCI/ADR**	Vincristine	None	500	--
		Verapamil-25 µM	0.83	1.4			Verapamil-10 µM	11	45.5
	Doxorubicin	None	50	--			Probenecid-100 µM	600	0.8
		Cyclosporin A-4 µM	150	0.3		Vinblastine	None	58	--
		Verapamil-20 µM	150	0.3			Verapamil-25 µM	1.7	34.1
	Cisplatin	None	7000	--		Paclitaxel	None	300	--
		Cyclosporin A-4 µM	17000	0.4			Verapamil-20 µM	5	60.0
		Verapamil-20 µM	10000	0.7		Doxorubicin	None	25000	--
MCF-7/VP	Vincristine	None	10	--			Cyclosporin A-4 µM	300	83.3
		Verapamil-10 µM	0.6	16.7			Verapamil-20 µM	400	62.5
		Probenecid-100 µM	4.5	2.2		Cisplatin	None	10000	--
	Paclitaxel	None	5	--			Cyclosporin A-4 µM	7000	1.4
		Verapamil-10 µM	5	1.0			Verapamil-20 µM	10000	1.0
	Doxorubicin	None	1000	--					
		Cyclosporin A-4 µM	1000	1.0					
		Verapamil-20 µM	2000	0.5					
	Cisplatin	None	15000	--					

Table 3.1. ***In vitro*** **cytotoxicity and reversal with modulators.** Four murine cell lines (JC, TIB-75, TIB-76, NMuLi) and three human breast adenocarcinoma cell lines [MCF-7; NCI/ADR (P-gp+ / MRP1-); MCF-7/VP (P-gp- / MRP1+) were evaluated with the indicated combinations of cytotoxic drugs and modulators. The reversal index is calculated as the ratio of the IC_{50} of the cytotoxic drug without modulator to the IC_{50} of the cytotoxic drug with modulator.

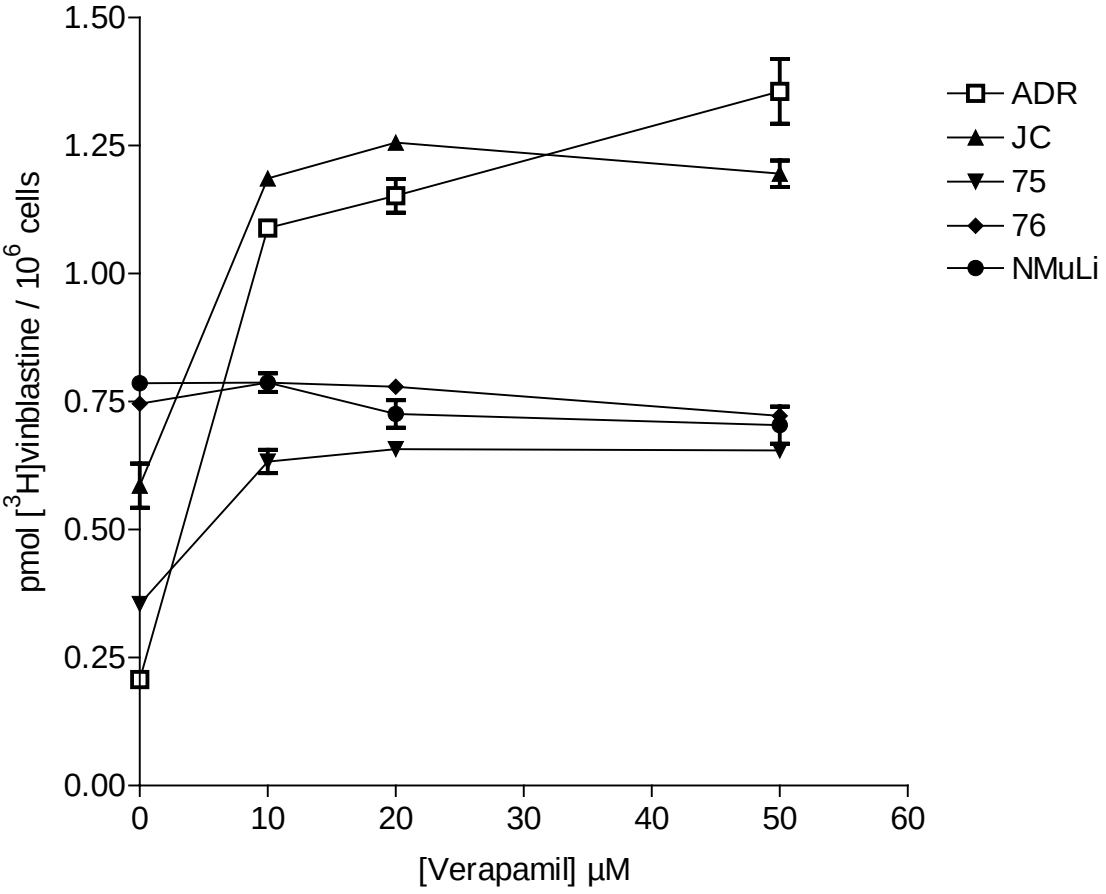
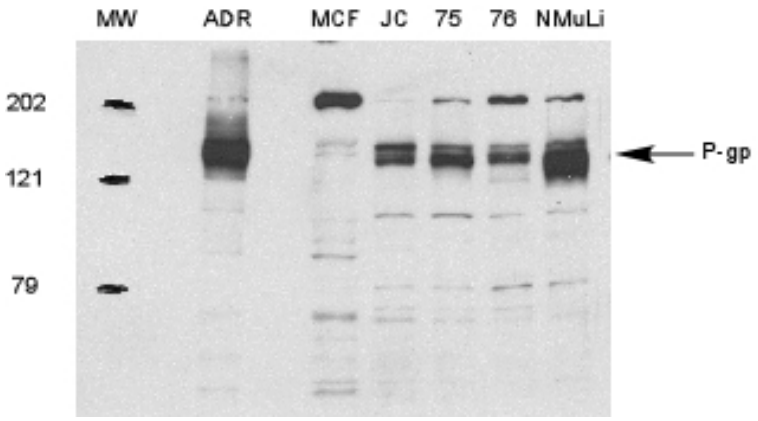


Figure 3.2. *In vitro* drug accumulation. Intracellular accumulation of [^{3}H]vinblastine was performed using JC (▲), TIB-75 (▼), TIB-76 (♦), NMuLi (●) or NCI/ADR (□) cells as described in "Materials and Methods." Results shown as mean ± SE for triplicate cultures.

reached at 20 μM verapamil, resulting in [^{3}H]vinblastine levels equivalent to approximately 200% of control. For comparison, [^{3}H]vinblastine accumulation by NCI/ADR cells, which greatly overexpress P-gp, was increased approximately 4-fold by 20 μM verapamil. In contrast, the TIB-76 and NMuLi cell lines accumulated greater amounts of [^{3}H]vinblastine in the absence of verapamil and did not show any increase in intracellular accumulation of [^{3}H]vinblastine when treated with verapamil. This is consistent with the results from the cytotoxicity assays, confirming that these cells do not display the MDR phenotype. [^{3}H]Taxol was also tested with the same cell lines and provided similar results (data not shown).

2. JC, TIB-75, TIB-76 and NMuLi cells show P-gp expression and no MRP1 expression. Membrane preparations from the four murine cell lines were prepared to determine the levels of P-gp expression in each of the murine cell lines. The MCF-7 human breast adenocarcinoma cell line, MCF-7/VP (P-gp - / MRP1+), and NCI/ADR (P-gp+ / MRP1 -) cells were used as controls for the Western blot analyses. As shown in Figure 3.3A, the expression of P-gp was seen in the JC and TIB-75 cell lines, which displayed the P-gp mediated-MDR phenotype *in vitro*. However, the TIB-76 and NMuLi cell lines also showed immunoreactivity, which does not correlate with the lack of a P-gp mediated MDR-phenotype displayed in the *in vitro* assays. This could be due to the lack of specificity of the antibody used in the Western analyses, in that it can cross-react with the mdr2 gene product, which has not been associated with drug efflux or the MDR phenotype (Gros et al., 1988). Alternatively, the P-gp expressed in the TIB-76 and NMuLi cell lines may be non-functional and therefore would not confer resistance to the

A.



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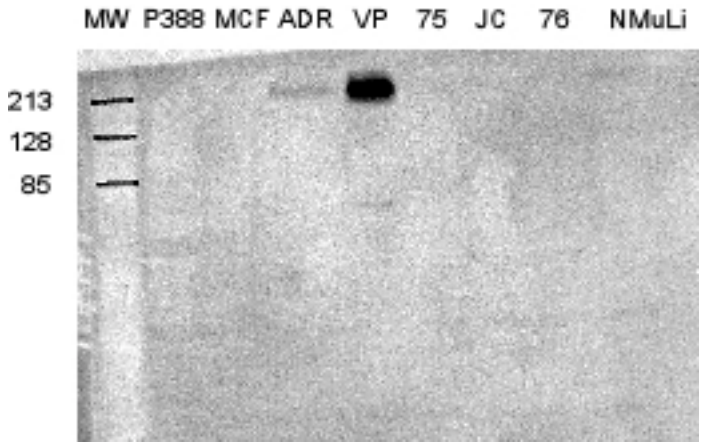


Figure 3.3. Western blot analysis. Membrane preparations were isolated from the indicated cell lines and Western blot procedures were conducted as described in "Materials and Methods." Filters were probed with (A) P-gp-specific antibody or (B) MRP1-specific antibody. Abbreviations include: molecular weight markers (MW), NCI/ADR human breast adenocarcinoma cells with P-gp expression (ADR), MCF-7 human breast adenocarcinoma cells (MCF), JC murine mammary adenocarcinoma cells (JC), NIB-75 murine liver cells (75), NIB-76 murine liver cells (76), normal murine liver epithelial cells (NMuLi), P388 murine lymphoid cells (P388), and MCF-7/VP human breast adenocarcinoma cells with MRP1 expression (VP).

classical P-gp substrates. As demonstrated in Figure 3.3B, only the MCF-7/VP cell line expressed MRP1, confirming that the MDR phenotype of the murine cell lines is mediated through the expression and function of P-gp.

3. *In vivo* validation of JC syngeneic solid tumor model. To analyze their *in vivo* growth kinetics and tumorgenicity, JC or TIB-75 cells were injected subcutaneously into female Balb/c mice, the strain from which the cell lines were originally derived. Both cell lines displayed excellent tumorgenicity with palpable tumors visible in approximately 2-3 weeks after inoculation, with *in vivo* take rates of greater than 95%. The JC cell line showed slightly more consistent growth than the TIB-75 cell line (data not shown). Therefore, the JC cell line was chosen for the subsequent validation of the syngeneic solid tumor model due to its spontaneous derivation (Chao and Chu, 1989), its *in vitro* expression of the MDR phenotype, and its reproducible *in vivo* growth.

To further characterize the JC *in vivo* solid tumor model and its effectiveness in evaluating MDR modulators, mice were inoculated with JC cells then subsequently treated with the cytotoxic drug, doxorubicin, with or without the known MDR modulator, cyclosporin A (Watanabe et al., 1995). As shown in Figure 3.4, mice in the control group experienced an increase in tumor volume of 700% by day 15, and animals were sacrificed because of the large size of the tumors. Animals treated with doxorubicin alone or cyclosporin A alone demonstrated increases in tumor size of 400% and 500%, respectively. In contrast, animals treated with the combination of doxorubicin and cyclosporin A experienced tumor growths of only 20% by day 15 ($P < 0.05$). In other replicates of this study, significant differences in the tumor volumes of the combination

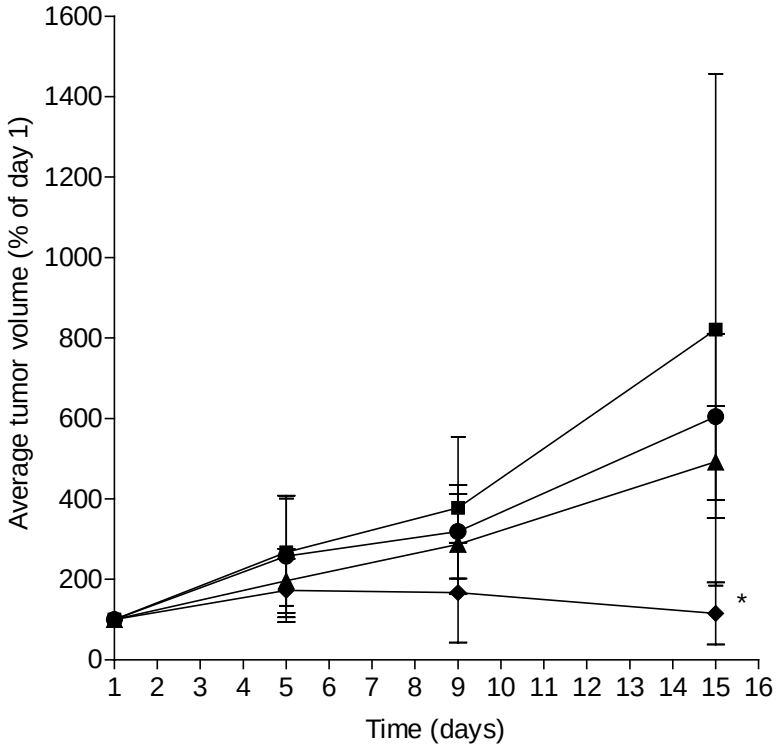


Figure 3.4. *In vivo* tumor model validation. Treatments of JC tumor-bearing female Balb/c mice with cyclosporin A and/or doxorubicin were performed as described in "Materials and Methods." Tumor volumes for control (■), doxorubicin-treated (▲), cyclosporin A-treated (●) or cyclosporin A plus doxorubicin-treated (♦) animals are shown as the mean ± SD (n = 4-5) of the percentage of the day 1 volume. *, $P < 0.05$.

group (doxorubicin and cyclosporin A) and the other three groups were manifested by day 9 and continued through the end of the study.

To determine whether P-gp expression was altered in the JC cells after systemic treatment with doxorubicin with or without cyclosporin A, the tumors were excised, minced and lysed on day 15 of the experiment. The tumor lysates were then analyzed for P-gp expression using Western blot techniques as previously described. The expression of P-gp was similar in all of the groups with no significant loss of expression in the JC tumors due to drug treatment (data not shown).

D. Discussion

Over the past 25 years, a number of *in vivo* models have been used to evaluate the efficacy of MDR modulators. Perhaps the most often used tumor model makes use of P388 mouse leukemia ascites and its P-gp expressing drug-resistant derivative, P388/ADR (Johnson et al., 1978; Tsuruo et al., 1981; Dantzig et al., 1996b). In this model, mice are injected intraperitoneally with the leukemia cells, then treated the same day with anticancer drug with or without a test modulator. In a typical experiment with verapamil and vinblastine, an increase in survival time of only 25-45% is seen with P388/ADR-bearing mice (van de Vrie et al., 1998). There are some advantages in utilizing this model to test the *in vivo* efficacy of MDR modulators, including low expense and good reproducibility. However, the intraperitoneal tumor model does not mimic human tumors and has low predictability since many modulators (i.e. verapamil)

that show efficacy in this model have failed in the clinic (van de Vrie et al., 1998). Furthermore, the treatment protocol of these animals does not mimic the treatment in a clinical setting, where a patient begins chemotherapy after a tumor mass has been discovered. Additionally, the direct administration of the drugs to the site of the tumor bypasses the vascular route used in clinical therapy. The vasculature of the tumor and the ability of the drugs to penetrate into the tumor are major factors in determining the success of the drug. Because of these limitations, the P388/ADR model can be useful for estimating the toxicity of a new modulator, but it has low value in estimating clinical efficacy. A somewhat better utilization of this model might involve administration of the modulator and cytotoxic drug via a different route, thereby requiring systemic delivery of these agents.

Another model to evaluate the *in vivo* efficacy of MDR modulators involves xenotransplantation of human tumor cells into immunocompromised animals (Plumb et al., 1994; Mistry et al., 1999). Perhaps the largest advantage of this model is the use of human tumor cells that express human P-gp. This allows for the modulation of human P-gp as compared to mouse P-gp expressed in the previously described P388/ADR model. Also, the results of experiments with xenograft models have been shown to be more relevant to clinical practice than those obtained from the P388 ascites model (Boven et al., 1992). Other advantages of xenograft models include the opportunity to delay treatment until palpable tumor formation, as well as the accessibility of the external tumors for serial measurements of volume. However, there are also several disadvantages to these models, including the expense and technical difficulties in using immunocompromised animals. Furthermore, the effect of the immune system on tumor formation and growth,

and the toxicity of the modulator to an intact host cannot be fully determined using immunocompromised animals.

A third option involves the use of syngeneic mouse cell lines that have been transfected with human *mdr1*, thereby expressing human P-gp. We have been unsuccessful in establishing a human *mdr1*-transfected murine solid tumor model due to the difficulty of expressing the large transmembrane protein. Currently available murine cell lines transfected with human *mdr1*, e.g. L1210/VMDRC.06, can only be used in an intraperitoneal *in vivo* model, thereby suffering from the same limitations as the P388 leukemia model. There would still be a number of potential disadvantages with a successful *mdr1*-transfected mouse solid tumor model. Since human P-gp is expressed, the murine immune system may respond to this foreign protein. Furthermore, alterations in tumorigenicity and growth rates and patterns have been described in *mdr1*-transfected cell lines (van de Vrie et al., 1998).

With the JC *in vivo* tumor model described herein, we sought to maintain the advantages of the previous models while limiting their disadvantages. This model allows for the rapid and inexpensive testing of P-gp modulators to determine their *in vivo* efficacy in inhibiting solid tumor growth using a highly tumorigenic cell line. Other advantages to this model include: treatment after palpable tumor formation; serial measurements; and the use of an intact animal to determine the effect of the immune system on the tumor and the toxicity of the modulator to the immune system. This model also uses an epithelial tumor cell line that was derived from a spontaneous primary mammary adenocarcinoma from a female Balb/c mouse (Chao and Chu, 1989). The JC cell line has not been chemically transformed or drug-selected. We have now

demonstrated that this cell line constitutively expresses endogenous P-gp and displays the MDR phenotype. Consequently, it may more closely resemble intrinsically resistant tumors found in a clinical setting than do the models discussed above.

The JC tumor model may also be more useful for pharmacological studies than other syngeneic *in vivo* models such as the Colon 26/MC26 murine colon carcinoma model. In the Colon26/MC26 model, the tumors are grown, excised, minced, and then re-implanted into the mice. Treatment typically begins 24-72 h after implantation, which does not allow for tumor adherence, growth and palpability (Spoelstra et al., 1991; Watanabe et al., 1995). There are clearly some limitations in using a syngeneic *in vivo* tumor model, including the present JC model. One disadvantage to this model is the fact that the JC cells are a murine cell line that expresses murine P-gp. Although there is greater than 80% amino acid sequence similarity between the murine *mdr1a/1b* and human *mdr1* gene products, there may be pharmacological differences between the proteins (Tan, 2000). It will therefore be critical to demonstrate that new modulators have similar activities towards human and murine P-gp via *in vitro* assays before committing to this model. Another limitation is the lack of an isogenic cell line that is drug-sensitive, which would allow for a more complete characterization of the reversal properties of new modulators.

We have previously described a number of natural products and synthetic compounds that effectively reverse MDR in tissue culture systems (Zilfou and Smith, 1995; Dinh et al., 1998; Smith et al., 2000; Lawrence et al., 2001; Xia and Smith, 2001). It is well documented that P-gp expression is significant in many tumors and a limited number of normal tissues, whereas MRP1 is widely expressed throughout normal tissues

(Ling, 1997; Sikic et al., 1997; Trock et al., 1997; Tan, 2000). This has led to the hypothesis that P-gp-specific modulators will be clinically superior to non-selective MDR modulators, since P-gp-targeted agents will be less likely to enhance the toxicity of the concomitantly administered anticancer drug to normal tissues.

In summary, we have developed a syngeneic *in vivo* tumor model that provides a valuable tool for the evaluation of P-gp modulators. Furthermore, we have shown its utility in demonstrating the *in vivo* efficacy of a known P-gp modulator, cyclosporin A, in combination with the cytotoxic drug, doxorubicin. This model will hopefully lead to a better predictive bridge between animal models and the clinic, and ultimately more successful development of MDR modulators.

CHAPTER 4

SYNTHESIS AND EVALUATION OF DIHYDROPYRROLOQUINOLINES THAT SELECTIVELY ANTAGONIZE P-GLYCOPROTEIN

A. Introduction

MDR is a phenomenon that leads to the failure of chemotherapy in cancer patients by allowing tumors to demonstrate resistance to a variety of structurally unrelated anticancer drugs. A major mechanism of MDR is through the overexpression of energy-dependent, unidirectional transmembrane efflux pumps. The drug transporter, P-glycoprotein (P-gp), is a 170 kDa protein that belongs to the ATP-binding cassette (ABC) superfamily of transporters (Juliano and Ling, 1976). Its biochemistry and pharmacology have been intensely studied for the past 25 years (Ling, 1997; Sikic et al., 1997; Tan, 2000). A series of homologous proteins termed multidrug resistance-related proteins (MRPs) have been discovered more recently, and these proteins share many pharmacological properties with P-gp (Grant et al., 1994; Kruh et al., 1994). The first protein of this series, MRP1, is a 190 kDa protein that was first identified in 1992 in a resistant lung cancer cell line that does not express P-gp (Cole et al., 1992). The ABC transporters function by binding to anticancer drugs within the cell and releasing them to

the extracellular space using energy from the hydrolysis of ATP (Horio et al., 1988). Tumor cells that are exposed to cytotoxic compounds often overexpress these efflux pumps, which allows these cells to survive when subsequently treated with anticancer drugs. The removal of the intracellular anticancer drugs spares the tumor cells from the effects of the drugs by not allowing interaction with their intracellular targets (Kartner et al., 1983).

MDR affects patients with a variety of cancers, both leukemias and solid tumors including breast, lung, and brain cancer. Overexpression of P-gp has been documented in a number of tumor types including acute leukemia and small-cell lung carcinoma, especially after the patient has received chemotherapy, indicating that this mechanism of MDR is clinically important (Bell et al., 1985; Fojo et al., 1987; Ma et al., 1987; Goldstein et al., 1989; Ling, 1997). Additionally, several studies have shown that P-gp expression can be a prognostic indicator in certain malignancies. For example, increased expression of P-gp in neuroblastoma and childhood sarcoma is associated with poor response to chemotherapy and decreased survival (van de Vrie et al., 1998), and breast cancer patients with P-gp expressing tumors are 3 times more likely to fail chemotherapy than those patients with P-gp negative tumors (Trock et al., 1997). In contrast, although MRP1 is expressed in a high percentage of leukemias and solid tumors (Nooter et al., 1995), its overexpression is not consistently found in tumors. For example, MRP1 levels detected in normal and malignant hematopoietic cells were equivalent (Abbaszadegan et al., 1994; Schneider et al., 1995), and the MRP1 levels in lung tumors were found to be lower than those in normal lung tissue (Thomas et al., 1994). The MRP1 mRNA levels in malignant melanoma (Schadendorf et al., 1995), acute lymphocytic leukemia (Hart et

al., 1994) or chronic lymphocytic leukemia (Leveille-Webster and Arias, 1995) were not altered by chemotherapy, but did increase moderately in acute myelogenous leukemia (Hart et al., 1994; Schneider et al., 1995). Therefore, it seems that overexpression of P-gp activity is clinically more significant than elevation of MRP1 levels.

Because of the importance of P-gp in clinical oncology for determining the success of chemotherapy, an intensive search has developed for antagonists of these transport proteins. These antagonists, often termed MDR modulators, function by blocking the transporter-mediated drug efflux so that a concomitantly administered anticancer drug can cause tumor cell death. Initial attempts to develop MDR modulators focused on the first generation drugs, verapamil and cyclosporin A (Tsuruo et al., 1981; Tan, 2000). These compounds demonstrated excellent *in vitro* reversal of MDR, but failed to achieve clinical success due to their intrinsic toxicity and/or their alteration of the pharmacokinetics of the co-administered anticancer drugs (Sikic et al., 1997; Kaye, 1998; Sikic, 1999). These clinical results may reflect differences in tissue distribution between the transport proteins, P-gp and MRP1.

Previous studies have shown that P-gp is expressed by certain types of secretory cells, including the capillary endothelial cells of the brain and testis, and by cells within the pancreas, kidney, liver and gastrointestinal tract (Endicott and Ling, 1989; Leveille-Webster and Arias, 1995). By comparison, mRNA of MRP1 has been observed in virtually every type of tissue within the body (Zaman et al., 1993) and is expressed in particularly high concentration in peripheral blood mononuclear cells (Cole et al., 1992; Abbaszadegan et al., 1994; Burger et al., 1994; Hart et al., 1994; Schneider et al., 1995).

The toxicity profile associated with the first generation MDR modulators may be due to inhibition of MRP1 in normal tissues.

The clinical significance of P-gp as well as its limited tissue expression as compared to MRP1 makes P-gp a more suitable drug target to reverse MDR. It is likely that the lack of ABC transporter-selectivity in early generation MDR modulators played a role in their ultimate failure. Therefore, we hypothesize that selectivity of individual MDR modulators to antagonize P-gp will lead to more clinically effective chemotherapeutic regimens. Some newer MDR modulators, including XR9576 (Martin et al., 1999; Stewart et al., 2000) and LY335979 (Dantzig et al., 1996b), have demonstrated improved P-gp selectivity and pharmacological properties (Roe et al., 1999; Newman et al., 2000a; Lawrence et al., 2001; Smith, 2001). To extend the portfolio of P-gp selective MDR modulators, we screened a library of compounds and discovered a series of dihydropyrroloquinolines that reverse P-gp-mediated MDR without antagonizing MRP1. We now describe the synthesis of substituted dihydropyrroloquinolines, structure-activity relationships for their effects on P-gp and MRP1, and the *in vivo* pharmacological properties of the most promising drug candidate, PGP-4008.

B. Materials and Methods

Reagents and starting materials were generally obtained from Aldrich Chemical (Milwaukee, MN) and used as received unless otherwise noted. Solvents were either

purchased as “anhydrous” or “ACS grade” and stored over 4 Å molecular sieves. “Flash chromatography” refers to the method of Still et al. (Still, 1978) and generally used Selecto Scientific silica gel (32-63 μm). Thin layer chromatography was performed on either BakerFlex IB-F or EM Science Silica Gel 60 F-254 plates (250 μm thick). Radial chromatography was performed on a Chromatotron Model 7924T (Harrison Research, Palo Alto, CA) using 1, 2, or 4 mm thick silica rotors. Melting points were determined in an open capillary on a MelTemp II melting point apparatus and are uncorrected. Infrared spectra were measured on a Nicolet Avatar 360 FT-IR; values are expressed in wavenumbers (cm^{-1}). ^{1}H- and ^{13}C-NMR spectra were obtained on a Bruker 200AM spectrometer. Chemical shifts are reported in ppm (δ) using tetramethylsilane (TMS) as reference and coupling constants (J) are reported in hertz. Mass spectra were obtained from Mass Consortium (San Diego, CA). Elemental analysis was performed by Midwest Microlab (Indianapolis, IN). All chemical yields are un-optimized and generally represent the result of a single experiment.

2-(1-Benzyl-pyrrolidin-2-ylideneamino)benzonitrile (30). Phosphorus oxychloride (5.2 mL) and tin(IV)chloride (1.0 mL) was added to a solution of chloroform (25 mL) and tetrahydrofuran (25 mL) containing 1-benzyl-2-pyrrolidinone (5.16 g, 29.4 mmol). The mixture was stirred at room temperature for 1.5 h, then anthranilonitrile (3.3 g, 27.9 mmol) was added in portions. The mixture was stirred at 50^{o}C for 5 h. After the reaction, ice-water (15 mL) was added and then a 30% aqueous sodium hydroxide solution was further added to make a weak alkaline. The organic solvent was removed under reduced pressure, and the mixture was extracted with chloroform. The extract was dried over anhydrous sodium sulfate and condensed under vacuum. The crude product was purified

by flash chromatography (chloroform-methanol, 100:1) to yield benzonitrile **30** (7.0 g, 91%) as slightly yellow needles, mp 59-61^{o}C; IR (KBr): 2217, 1628, 1439, 1279cm^{-1}; ^{1}H NMR (DMSO-d_6): 7.06-7.69 (m, 9H), 4.83 (s, 2H), 3.46 (t, 2H), 2.60 (t, 2H), 2.10 (m, 2H); ^{13}C NMR ($CDCl_3$): 162.8, 156.3, 137.6, 133.7, 132.9, 128.7(2C), 128.3(2C), 127.4, 122.8, 121.7, 118.8, 105.9, 48.3, 47.3, 27.7, 19.6. Anal. Calc. for $C_{18}H_{17}N_3$(275.35): C, 78.52; H, 6.22; N, 15.26. Found: C, 78.37; H, 6.20; N, 15.17.

1-Benzyl-2,3-dihydro-*1H*-pyrrolo[2,3-b]quinolin-4-ylamine (18). A solution of tetrahydrofuran (350 mL) containing **30** (37.6 g, 0.14 mol) was cooled to -35^{o}C under argon atmosphere, and then 1.5M lithium diisopropylamine-tetrahydrofuran complex (233 mL, 0.35 mol) in cyclohexane was added dropwise. After the addition was complete, the temperature of the mixture was gradually raised to -10^{o}C, and ice-water (30 mL) was added dropwise. After the organic solvent was evaporated under reduced pressure, the mixture was extracted with chloroform. The combined organic extracts were dried over anhydrous sodium sulfate and condensed under vacuum. The residue was treated with ethanol (15 mL) and crystals were precipitated. The precipitate was filtered, washed with cold ethanol and dried under vacuum, which yielded 14.6 g (39%) of ylamine **18** as needles, mp 174-176^{o}C; IR (KBr): 3411, 3116, 1654, 1502, 1350, 756cm^{-1}; ^{1}H NMR (DMSO-d_6): 7.01-7.93 (m, 9H), 6.05 (s, 2H), 4.60 (s, 2H), 3.37 (t, 2H), 2.87 (t, 2H); ^{13}C NMR ($CDCl_3$): 162.7, 149.3, 144.8, 138.8, 128.7(2C), 128.2(2C), 128.1, 127.2, 126.2, 121.8, 119.9, 117.6, 100.7, 48.4(2C), 23.3. Anal. Calc. for $C_{18}H_{17}N_3$(275.35): C, 78.52; H, 6.22; N, 15.26. Found: C, 78.07; H, 6.07; N, 15.08.

General Procedure for the synthesis of compounds 31-34. To a solution of **18** (1 mmol) in tetrahydrofuran (15 mL), potassium *tert*-butoxide was added at 0^{o}C under

nitrogen atmosphere. After stirring at room temperature for 1.5 h, the reaction mixture was added dropwise to a solution of corresponding alkyl chloride (1 mmol) in tetrahydrofuran (10 mL) via syringe at -5°C. The reaction mixture was warmed to room temperature and stirred for 1.5-3 h. The reaction mixture was then poured into water (50 mL) and solid was precipitated. The precipitate was filtered and dried under vacuum. The crude product was purified by flash silica gel chromatography (chloroform-methanol, 100:1) to give the corresponding product.

(1-Benzyl-2,3-dihydro-*1H*-pyrrolo[2,3-b]quinolin-4-ylamino)-acetic acid ethyl ester (31). Yield 61%; mp 132-134°C; IR (KBr): 3410, 1745, 1620, 1502, 1215cm^{-1}; ^{1}H NMR ($CDCl_3$): 7.16-7.72 (m, 9H), 4.77 (s, 2H), 4.25 (q, 2H), 3.51 (t, 2H), 3.49 (s, 2H), 3.11 (t, 2H), 1.34 (t, 3H); ^{13}C NMR ($CDCl_3$): 171.1, 157.4, 139.6, 137.1, 135.5, 132.1, 128.8(2C), 128.5(2C), 128.3, 127.3, 126.5, 124.5, 121.7, 120.5, 61.9, 48.7, 48.0, 26.1, 24.9, 14.6. Anal. Calc. for $C_{22}H_{23}N_3O_2$ (361.18): C, 73.11; H, 6.41; N, 11.63. Found: C, 73.04; H, 6.08; N, 11.90.

(1-Benzyl-2,3-dihydro-*1H*-pyrrolo[2,3-b]quinolin-4-yl)-(3-fluoro-benzyl)-amine (32). Yield 65%; mp 170-171°C; IR (KBr): 3400, 3063, 1622, 1504, 1217cm^{-1}; ^{1}H NMR ($CDCl_3$): 7.00-8.15 (m, 13H), 4.73 (s, 2H), 4.19 (s, 2H), 3.21 (t, 2H), 2.45 (t, 2H); ^{13}C NMR ($CDCl_3$): 164.7, 162.5, 159.8, 149.8, 148.6, 137.8, 136.4, 133.5, 133.4, 130.4, 130.2, 128.6, 128.4, 128.2, 127.2, 126.6, 123.6, 121.7, 119.1, 115.4, 115.0, 54.5, 48.8, 47.8, 25.4. Anal. Calc. for $C_{25}H_{22}FN_3$ (383.46): C, 78.30; H, 5.78; N, 10.96. Found: C, 78.23; H, 5.32; N, 11.21.

(1-Benzyl-2,3-dihydro-*1H*-pyrrolo[2,3-b]quinolin-4-yl)-(4-fluoro-benzyl)-amine (33). Yield 80%; mp 167-169°C; IR (KBr): 3405, 1620, 1502, 1215cm^{-1}; ^{1}H NMR ($CDCl_3$):

6.92-8.10 (m, 13H), 4.72 (s, 2H), 4.21 (s, 2H), 3.21 (t, 2H), 2.48 (t, 2H); ^{13}C NMR ($CDCl_3$): 165.5, 162.5, 160.6, 149.9, 148.5, 140.3, 137.8, 129.9, 128.7, 128.5, 128.2, 127.2, 126.7, 124.3, 123.5, 121.8, 119.1, 115.7, 115.3, 114.7, 114.2, 54.9, 48.9, 47.9, 25.5. Anal. Calc. for $C_{25}H_{22}FN_3$ (383.46): C, 78.30; H, 5.78; N, 10.96. Found: C, 78.73; H, 5.75; N, 11.08.

(1-Benzyl-2,3-dihydro-*1H*-pyrrolo[2,3-b]quinolin-4-yl)-(3,5-dimethoxy-benzyl)-amine (34). Yield 84%; mp 133-135^{o}C; IR (KBr): 3420, 1621, 1509, 1218cm^{-1}; ^{1}H NMR ($CDCl_3$): 6.57-8.15 (m, 12H), 4.71 (s, 2H), 4.13 (s, 2H), 3.67 (s, 6H), 3.23 (t, 2H), 2.56 (t, 2H); ^{13}C NMR ($CDCl_3$): 162.1, 160.6(2C), 149.5, 149.0, 141.4, 139.2, 1291, 128.4(2C), 128.2(2C), 127.2, 126.5, 123.7, 121.7, 121.5, 119.8, 106.6, 106.3, 99.6, 55.6(2C), 55.2, 48.8, 47.9, 25.5. Anal. Calc. for $C_{27}H_{27}N_3O_2$ (425.52): C, 76.21; H, 6.40; N, 9.87. Found: C, 76.23; H, 6.36; N, 9.50.

General Procedure for the synthesis of compounds 27 and 35-38. To a suspension of sodium hydride (1 mmol) in DMF (20 mL) at 0^{o}C under nitrogen atmosphere, a solution of **18** (1 mmol) in DMF (10 mL) was added dropwise. After 5 min, a corresponding acyl chloride was slowly added via syringe over a 20 min period at –5^{o}C. The reaction mixture was stirred at room temperature for 1-4 h (Table 2), then filtered through a pad of silica gel. The solvent was removed under reduced pressure, and the residue was purified by flash silica gel chromatography (chloroform-methanol, 100:1) to give the corresponding product.

***N*-(1-Benzyl-2,3-dihydro-*1H*-pyrrolo[2,3-b]quinolin-4-yl)-2-piperidin-1-yl-acetamide (27).** Yield 49%; mp 220-222^{o}C; IR (KBr): 3410, 1699, 1660, 1505cm^{-1}; ^{1}H NMR (DMSO-d_6): 7.05-7.78 (m, 9H), 4.60 (s, 2H), 4.46 (s, 2H), 3.46 (m, 2H), 3.10 (m, 4H),

2.86 (t, 2H), 1.72 (m, 4H), 1.52 (m, 2H); ^{13}C NMR (DMSO-d_6): 168.6, 163.6, 149.6, 145.9, 139.5, 129.9(2C), 129.7(2C), 129.5, 128.6, 126.6, 122.7, 121.9, 118.4, 102.4, 60.6, 54.5(2C), 49.5(2C), 24.1, 23.9(2C), 22.7. Anal. Calc. for $C_{25}H_{28}N_4O.HCl$ (437.02): C, 68.70; H, 6.70; N, 12.81. Found: C, 68.81; H, 6.43; N, 12.76.

***N*-(1-Benzyl-2,3-dihydro-*1H*-pyrrolo[2,3-b]quinolin-4-yl)-2-phenyl-acetamide (35 or PGP-4008).** Yield 52%; mp 157-159^{o}C; IR (KBr): 3431, 2959, 1690, 1665, 1507, 1310cm^{-1}; ^{1}H NMR (DMSO-d_6): 7.29-8.37 (m, 14H), 5.17 (s, 2H), 3.93 (s, 2H), 3.77 (t, 2H), 2.97 (t, 2H); ^{13}C NMR (DMSO-d_6): 169.5, 156.5, 139.4, 137.2, 136.4, 135.1, 131.7, 129.9, 129.5(2C), 129.1(2C), 129.0(2C), 128.8(2C), 127.4, 125.3, 124.6, 123.6, 119.9, 118.8, 51.0, 50.5, 43.1, 25.8. Anal. Calc. for $C_{26}H_{23}N_3O.H_2O$ (411.50): C, 75.88; H, 6.13; N, 10.21. Found: C, 76.29; H, 6.41; N, 9.82.

***N*-(1-Benzyl-2,3-dihydro-*1H*-pyrrolo[2,3-b]quinolin-4-yl)-2-fluoro-6-trifluoromethyl-benzamide (36).** Yield 55%; mp 189-191^{o}C; IR (KBr): 3427, 2933, 1691, 1628, 1119cm^{-1}; ^{1}H NMR (DMSO-d_6): 7.35-8.25 (m, 12H), 5.02 (s, 2H), 3.66 (t, 2H), 2.98 (t, 2H); ^{13}C NMR (DMSO-d_6): 168.5, 165.3, 164.8, 161.2, 156.4, 148.4, 138.6, 136.4, 134.5, 131.1, 129.3(2C), 128.7(2C), 128.3, 124.1, 123.3, 122.5, 119.4, 119.1, 118.6, 116.3, 99.7, 50.3, 49.8, 23.8. Anal. Calc. for $C_{26}H_{19}F_4N_3O$ (465.44): C, 67.09; H, 4.11; N, 9.03. Found: C, 67.45; H, 4.19; N, 9.15.

***N*-(1-Benzyl-2,3-dihydro-*1H*-pyrrolo[2,3-b]quinolin-4-yl)-4-fluoro-3-trifluoromethyl-benzamide (37).** Yield 45%; mp 195-197^{o}C; IR (KBr): 3435, 1691, 1626, 1375, 1118cm^{-1}; ^{1}H NMR (DMSO-d_6): 7.25-8.62 (m, 12H), 4.95 (s, 2H), 3.37 (t, 2H), 3.00 (t, 2H); ^{13}C NMR (DMSO-d_6): 169.0, 165.0, 164.9, 161.2, 155.4, 147.9, 138.8, 136.0, 134.3, 131.0, 129.1, 128.7(2C), 128.2(2C), 124.1, 123.1, 122.5, 119.4, 119.1,

118.3, 116.7, 99.5, 50.8, 49.5, 23.5. Anal. Calc. for $C_{26}H_{19}F_4N_3O$ (465.44): C, 67.09; H, 4.11; N, 9.03. Found: C, 67.50; H, 4.05; N, 9.18.

***N*-(1-Benzyl-2,3-dihydro-*1H*-pyrrolo[2,3-b]quinolin-4-yl)-2,3,6-trifluoro-benzamide (38).** Yield 53%; mp 160-162°C; IR (KBr): 3430, 1689, 1378, 1119cm^{-1}; ^{1}H NMR (DMSO-d_6): 7.33-8.31 (m, 11H), 5.14 (s, 2H), 3.84 (t, 2H), 3.13 (t, 2H); ^{13}C NMR (DMSO-d_6): 168.9, 165.3, 164.8, 161.2, 159.2, 159.1, 159.0, 138.2, 135.6, 132.3, 130.0, 129.4, 129.3, 125.9, 125.6, 124.5, 120.9, 120.5, 120.2, 119.6, 113.9, 113.4, 50.7, 49.8, 23.6. Anal. Calc. for $C_{25}H_{18}F_3N_3O$ (433.43): C, 69.28; H, 4.19; N, 9.69. Found: C, 69.28; H, 4.07; N, 9.62.

***N*-(1-Benzyl-2,3-dihydro-*1H*-pyrrolo[2,3-b]quinolin-4-yl)-2-phenyl-acetamide hydrochloride salt (35 or PGP-4008-2HCl).** PGP-4008 (35), 400 mg, was suspended in 1M HCl in ether (8 mL). The mixture was stirred at room temperature for 12 h. The solvent was removed with nitrogen flushing. The residue was washed with 10% EtOAc in hexane twice and dried *in vacuo* to afford the yellowish solid, PGP-4008-2HCl salt, 470 mg (Yield=99%). ^{1}H NMR (DMSO-d_6): 11.03 (s, 1H), 7.20-8.20 (m, 14H), 5.13 (s, 2H), 3.89 (s, 2H), 3.74 (t, 2H), 2.94 (t, 2H); ^{13}C NMR (DMSO-d_6): 168.5, 155.7, 138.4, 135.4, 134.2, 130.7, 129.1, 128.9 (2C), 128.6 (2C), 128.1 (2C), 128.0 (2C), 127.8, 126.4, 124.2, 123.6, 122.5, 119.0, 118.0, 50.0, 49.5, 42.2, 24.8.

***In vitro* cytotoxicity assay.** MCF-7 and NCI/ADR cells were obtained from the Division of Cancer Treatment of the National Cancer Institute.(Fairchild et al., 1987a) MCF-7/VP cells were provided by Drs. Schneider and Cowan.(Schneider et al., 1994) Cells were maintained in RPMI 1640 (Life Technologies, Inc., Rockville, MD) with L-glutamine containing 10% FBS and 50 μg/mL gentamicin at 37°C and 5% CO_2 in 100 mm x 20

polystyrene tissue culture dishes. Cells were seeded in 96-well tissue culture dishes at approximately 20% confluency and allowed to recover and attach for 24 h. Cells were then treated with varying concentrations of modulators (as allowed by solubility) in the presence or absence of cytotoxic drugs for 48 h. The number of surviving cells remaining in each well was quantitated with the sulforhodamine B (SRB) colorimetric assay (Skehan et al., 1990). Briefly, cells were washed with phosphate-buffered saline (PBS) and fixed to the plate with 10% trichloroacetic acid. The cells were then washed with water and stained with 0.4% SRB in 1% acetic acid. Cells were then rinsed with 1% acetic acid and 10 mM Tris buffer was added to dissolve the SRB. The absorbance of each well was determined with a PerkinElmer HTS 7000 Plus BioAssay plate reader at a wavelength of 570 nm. The percentage of cells killed is calculated as the percentage decrease in SRB binding as compared with control cultures, and is taken from the mean of the absorbance measurements of three equally treated wells. Reversal of P-gp-mediated MDR is indicated if the compound enhances the toxicity of vinblastine toward the NCI/ADR cells. The P-gp Antagonism Score is calculated as the percentage of surviving NCI/ADR cells in the absence of vinblastine / the percentage of surviving NCI/ADR cells in the presence of vinblastine. Control cultures included equivalent amounts of ethanol (as the solvent control), which did not modulate the growth or drug-sensitivity of these cells at the doses used in these studies. To assess the toxicity of the compounds toward drug-sensitive cells, the effects of the test modulators on the growth of drug-sensitive MCF-7 cells were determined by the same methods. Reversal of MRP1-mediated MDR is indicated if the compound enhances the toxicity of vincristine toward MCF-7/VP cells. The MRP1 Antagonism Score is calculated as the percentage of

surviving MCF-7/VP cells in the absence of vincristine / the percentage of surviving MCF-7/VP cells in the presence of vincristine.

***In vitro* drug accumulation assay.** Cells were seeded in 24-well tissue culture dishes at approximately 25% confluency and allowed to recover and grow to near confluency, approximately 3-4 days. Media was aspirated and replaced with serum-free media. PGP-4008, dissolved in 100% ethanol, was incubated for 30 min. at 37°C. Approximately 0.1 μCi of [^{3}H]Taxol (75 Ci/mmol) or [^{3}H]vinblastine (7.3 Ci/mmol) (Moravek Biochemicals, Brea, CA) was then added per well and the cultures were incubated for 60 min. at 37°C. Radioactive media was aspirated and cells were rapidly washed twice with ice-cold PBS. Intracellular [^{3}H]drug was solubilized with 1% sodium dodecyl sulfate (SDS) in water and quantified by liquid scintillation counting using UniverSol (ICN, Costa Mesa, CA) (Smith et al., 1995).

***In vivo* toxicity.** Animal care and procedures were in accordance with guidelines and regulations of the Institutional Animal Care and Use Committee of The Penn State College of Medicine. Female Swiss-Webster mice (Charles River Laboratories, Wilmington, MA), 6-8 weeks old, were acclimated to their environment during quarantine for approximately 10 days before being released into the mouse colony. Mice were housed (n=5 per cage) under 12 h light/dark cycles with food and water provided *ad libitum*. To observe the *in vivo* toxicity of PGP-4008, mice were injected in the intraperitoneal (i.p.) cavity with 100 μL of DMSO containing 20 mg/mL of PGP-4008 to give a total body concentration of approximately 100 mg/kg (2.0 mg total dose). Doses were administered once a day for 5 consecutive days. Mice were observed for 2-3 weeks after the final injection.

***In vivo* solid tumor growth inhibition.** Balb/c female mice (Charles River Laboratories, Wilmington, MA), 6-8 weeks old, were injected in the subcutaneous space of the right hind quarter with 10^6 JC cells (murine mammary adenocarcinoma, American Type Culture Collection CRL-2116, Manassas, VA) suspended in PBS. After palpable tumor growth, approximately 2-3 weeks after injection, tumor volume was determined (day 1) using calipers measuring the length (L) and width (W) of the tumor. Tumor volume was calculated using the equation: $(L \times W^2)/2$, and animals were randomized into four groups (n=5 per group). Treatment was then administered on days 1, 5, and 9, and consisted of either an intravenous (i.v.) administration of a non-specific MDR modulator, cyclosporine injection USP (Bedford Laboratories, Bedford, OH) at a dose of 50 mg/kg, with or without the cytotoxic drug, doxorubicin hydrochloride (Sigma-Aldrich Co., St. Louis, MO) at a dose of 5 mg/kg or an i.p. administration of PGP-4008 hydrochloride salt at a dose of 100 mg/kg with or without the i.v. administration of doxorubicin hydrochloride at a dose of 5 mg/kg. Tumor volumes were monitored until day 15 when the animals were euthanized (Watanabe et al., 1995). Unpaired t-test with Welch correction was performed using GraphPad InStat version 3.01 for Windows 95 (GraphPad Software, San Diego, CA).

***In vivo* pharmacokinetics.** Three female Swiss-Webster mice, 6-8 weeks old, were injected i.p. with approximately 100 mg/kg of PGP-4008 in a volume of 100 μL DMSO. At each of the following time points: 1, 5, 10, 15, 30 and 45 minutes, as well as 2, 3 and 4 hours after injection, the mice were anesthetized with 2-bromo-2-chloro-1,1,1-trifluoroethane (Halothane, Sigma-Aldrich, St. Louis, MO) and blood samples (700-800 μL) were extracted by intracardiac puncture. The mice were then euthanized by cervical

dislocation. All samples were immediately weighed and stored at –20°C. Samples were then prepared by adding 1 mL of cold 0.9% saline to each tissue sample and homogenized via sonication. After homogenization, the internal standard, 2-naphthol, was added. Each sample was then precipitated with 12 mL ice-cold acetonitrile, vortexed vigorously, and centrifuged at 4000 x g for 15 minutes at 4°C. The acetonitrile layer was then transferred to glass test tubes and evaporated to dryness over a stream of nitrogen using the N-Evap concentration system (Organomation, Berlin, MA) with the water bath set at 45 °C. Samples were reconstituted in 200 μL of methanol and either analyzed immediately or stored at -20°C. Samples were analyzed using a Beckman HPLC System Gold module. Samples were injected into a 20 μL loop and resolved on a C8 reverse phase Ultrasphere column (4 μm particle size, 4 x 250 mm, Beckman) with an isocratic mobile phase consisting of 60% H_2O + 0.1% trifluoroacetic acid and 40% methanol (HPLC grade, Fisher). The flow rate was 1 mL/min with an average pressure of 2500 psi. Internal standard and PGP-4008 elution were monitored at 254 nm with elution times of 7.9 min and 14.3 min, respectively. Peaks were integrated and PGP-4008 concentrations were determined by using a standard curve, which was linear in all area ratios observed ($r^2 = 0.995$). The relative recoveries and coefficients of variation (CV) for the intraassay accuracy and precision were 92–106% and 4–8%, respectively. The quantitation limit was 0.2 ng /mL. Interassay accuracy and precision were similar, with relative recovery and coefficient of variation values of 91–110% and 7–12%, respectively. All concentration values in figures represent the mean of replicates from a single typical experiment, with the bars representing the SEM. All pharmacokinetic analyses were performed using the WinNonLin Standard software package (Pharsight). The area under

the curve (AUC) was determined using the linear trapezoidal rule. The first order elimination rate constant (k_{el}) was calculated using the slope of linearized log concentration plot. Elimination half-life was determined by the formula $k_{el} = \alpha\beta/k_{21}$. The bioavailability (F) following intraperitoneal administration of Pgp-4008 was determined by the following formula: $F= (AUC_{IP}/AUC_{IV}) * 100$.

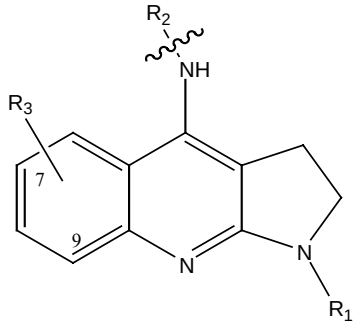
***In vivo* pharmacokinetics of coadministered anticancer drugs.** Three female Swiss-Webster mice, 6-8 weeks old, were injected i.p. with approximately 100 mg/kg of PGP-4008 in a volume of 100 μL DMSO. Approximately 5 minutes after i.p. injection, the mice were injected i.v. with [^{3}H]vinblastine (~ 2 μCi/animal) at a dose of 10 mg/kg in a volume of 100 μL 0.9% saline. At each of the following time points: 0.5, 1, and 2 hours after i.v. injection, the mice were anesthetized with 2-bromo-2-chloro-1,1,1-trifluoroethane (Halothane, Sigma-Aldrich, St. Louis, MO) and blood samples (700-800 μL) were extracted by intracardiac puncture. The mice were then euthanized by cervical dislocation. All samples were immediately weighed and stored at –20°C. Samples were then prepared by adding 1 mL Solvable (Packard Bioscience Co., Meriden, CT) and incubated at 50°C for 1 h. 0.1 mL of 100 mM EDTA and 0.3 mL of 30% H_2O_2 was then added to decolorize the samples. Samples were incubated for 1 h at 50°C, allowed to cool to room temperature before the addition of 15 mL of UniverSol (ICN, Costa Mesa, CA) and quantitated by liquid scintillation counting (Saito et al., 2001).

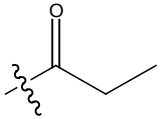
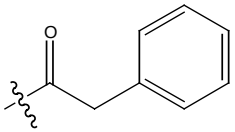
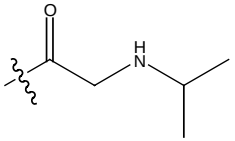
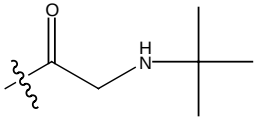
C. Results and Discussion

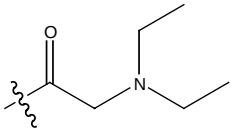
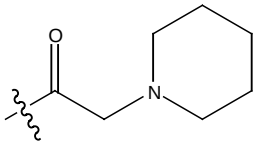
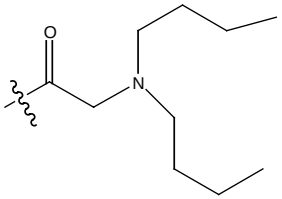
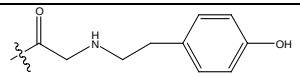
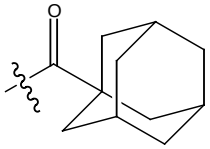
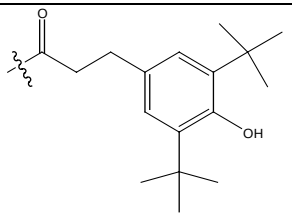
1. Identification of MDR-reversing dihydropyrroloquinolines. As with our previous studies (Smith et al., 1995; Lawrence et al., 2001), we sought new inhibitors of P-gp and MRP1 using cell-based cytotoxicity assays with the following tumor cell lines: MCF-7, a drug-sensitive human breast adenocarcinoma line; NCI/ADR, a line selected for resistance to adriamycin that expresses high levels of P-gp without the overexpression of MRP1; and MCF-7/VP, a subline of MCF-7 selected for resistance to etoposide in the presence of verapamil that expresses high levels of MRP1 without the overexpression of P-gp. Assays were conducted such that the cells were treated with a P-gp-substrate drug, vinblastine, or an MRP1-substrate drug, vincristine, either alone or in the presence of a test MDR modulator. Verapamil is used as the positive control in all of our studies and is highly effective at reversing P-gp and MRP1-mediated MDR *in vitro*.

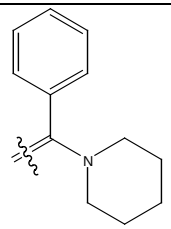
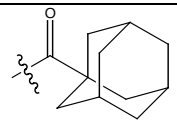
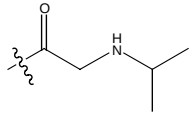
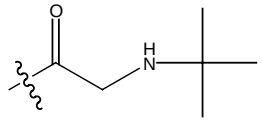
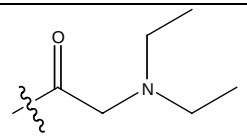
Screening approximately 11,000 compounds from a commercially available library identified several structural platforms with good potential as MDR antagonists. One of these, the dihydropyrroloquinoline heterocycle, showed promise as a versatile scaffold with which to study structure-activity relationships (SARs) for inhibition of P-gp activity. The initial screening was conducted at a single final dose of 10 μg/mL for the test compounds, equating to concentrations of approximately 15 - 25 μM. The library contained 29 dihydropyrroloquinolines, whose biological activities are described in Table 4.1. Firstly, the intrinsic cytotoxicity of the compounds toward MCF-7 cells is indicated. Toxicity is expressed as the percentage of MCF-7 cells killed by 10 μg/ml of the indicated compound. It can be seen that these compounds have a wide range of toxicity, varying from 0% of cells killed by Compound **27** to 99% of cells killed by Compounds

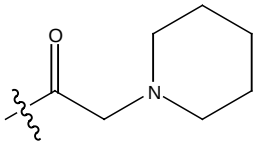
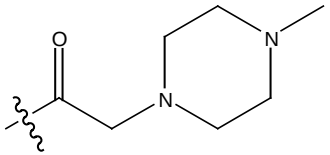
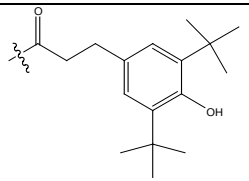
Table 4.1. Cytotoxicity and MDR antagonism by substituted dihydropyrroloquinolines.



compd	R_1	R_2	R_3	Toxicity[a]	P-gp[b]	MRP1[c]	P-gp/MRP1
1	methyl	H	7-Br	94	1.3	1.6	0.8
2	methyl			12	1.6	1.0	1.6
3	methyl			22	5.6	1.0	5.6
4	methyl			39	1.8	1.4	1.3
5	methyl			28	3.2	1.2	2.7

6	methyl			30	6.0	1.2	5.0
7	methyl			51	4.1	1.2	3.4
8	methyl			50	4.2	1.3	3.2
9	methyl			21	1.7	1.4	1.2
10	methyl			29	4.7	0.9	5.2
11	methyl			99	1.0	1.0	1.0

12	methyl			58	2.4	1.0	2.4
13	butyl	H	7-Br	98	1.1	1.0	1.1
14	cyclohexyl	H		97	0.8	0.6	1.3
15	cyclohexyl	H	7-Br	99	1.0	1.0	1.0
16	phenyl	H		52	2.7	1.1	2.5
17	3-chloro-benzyl			33	1.2	1.2	1.0
18	benzyl	H		47	3.1	1.0	3.1
19	benzyl	H	7-Br	94	1.7	1.3	1.3
20	benzyl	H	7-CH_3	98	0.7	1.0	0.7
21	benzyl	H	9-CH_3	77	1.6	1.7	0.9
22	benzyl	H	6,9-CH_3	81	1.5	1.2	1.3
23	benzyl	H	7,9-CH_3	49	3.6	1.4	2.6
24	benzyl			45	13	1.0	13
25	benzyl			29	12	0.9	13
26	benzyl			14	15	1.0	15

27	benzyl			0	18	1.1	16
28	benzyl			21	7.5	1.1	6.8
29	benzyl			50	13.2	1.2	11

[a] Toxicity is calculated as the percentage of MCF-7 cells killed by 10 μg/mL of the indicated compound.

[b] The P-gp antagonism score = percentage of NCI/ADR cells surviving in the absence of vinblastine / percentage of NCI/ADR cells surviving in the presence of vinblastine.

[c] The MRP1 antagonism score = percentage of MCF-7/VP cells surviving in the absence of vincristine / percentage of MCF-7/VP cells surviving in the presence of vincristine.

11 and **15**. While toxicity toward cultured cancer cells is a typical and desired property for drugs with utility in the therapy of cancer, it is desirable that chemosensitizing compounds have low intrinsic toxicity. Secondly, the ability of the compounds to reverse P-gp-mediated drug resistance is indicated in the same table. The P-gp antagonism score is calculated as the percentage survival of NCI/ADR cells treated with the compound alone divided by the percentage survival of NCI/ADR cells treated with the compound plus 50 nM vinblastine. Therefore, since an antagonism score of 1.0 indicates inactivity of the test compound while larger antagonism scores indicate increasing activity, it is apparent that several of the dihydropyrroloquinolines demonstrate this property. Thirdly, the ability of the compounds to reverse MRP1-mediated drug resistance is indicated. As above, the MRP1 antagonism score is calculated as the percentage survival of MCF-7/VP cells treated with the compound alone divided by the percentage survival of MCF-7/VP cells treated with the compound plus 1 nM vincristine, so that chemosensitization is indicated by a score greater than 1.0. In general, the dihydropyrroloquinolines had only marginal abilities to reverse MRP1-mediated MDR, so that several compounds are effective inhibitors of P-gp without inhibiting the action of MRP1. For example, Compound **27** has a P-gp antagonism score of 18 whereas the MRP1 antagonism score is only 1.1. Compounds having a benzyl substitution at R_1 were more active toward P-gp than were corresponding compounds with methyl groups at that site, e.g. Compound **27** compared with Compound **7** and Compound **29** compared with Compound **11**. Additionally, increasing the size and/or hydrophobicity of the substituent at position R_2 enhanced activity toward P-gp, e.g. Compound **27** > **26** > **25** = **24**. Thus, the initial

screening indicated that bisubstituted dihydropyrroloquinolines provide a new and versatile chemotype for the development of P-gp-selective MDR antagonists.

2. Synthesis and *in vitro* evaluation of novel dihydropyrroloquinolines. A series of substituted dihydropyrroloquinolines, shown in Table 4.2, was synthesized as described in Scheme 1. 2-Amino-benzonitrile reacted with 1-benzyl-2-pyrrolidinone in the presence of phosphorus oxychloride and tin(IV)chloride to yield 2-(1-benzyl-pyrrolidin-2-ylidenemethyl)-benzonitrile (**30**). Following the addition of lithium diisopropylamine-tetrahydrofuran complex, 1-benzyl-2,3-dihydro-1H-pyrrolo[2,3-b]quinolin-4-ylamine (**18**) was produced. Through alkylation and acetylation reactions, compounds **31-34**, **27**, and **35-38** were obtained. For *in vivo* studies described below, **35** or PGP-4008 was reacted with 2 equivalents of hydrogen chloride in ether to obtain the corresponding HCl salt.

As detailed in Table 4.3 and Figure 4.1, the newly synthesized dihydropyrroloquinolines were found to exhibit a broad range of activity against P-gp, with PGP-4008 having a maximal activity equivalent to verapamil at a significantly lower dose (Figure 4.1A). More importantly, none of the dihydropyrroloquinolines, including PGP-4008, displayed any significant antagonism towards MRP1 (Figure 4.1B).

PGP-4008 was further characterized in a variety of cell lines with different cytotoxic drugs (Table 4.4). The Reversal Index (RI) is calculated as the ratio of the IC_{50} in the absence of modulator / the IC_{50} in the presence of modulator, so that larger values indicate increasing activity. PGP-4008 potentiated the cytotoxicity of P-gp substrate drugs, including vinblastine, vincristine and paclitaxel, toward cell lines that overexpress

Table 4.2. Reaction conditions, yields, and melting points of compounds **27** and **31-38**.

Products	Reaction Conditions		Yield	mp
	Cat [a]	Time(h)	(%)[b]	(^{o}C)
31	A	1.5	61	132-134
32	A	2	65	170-171
33	A	3	80	167-169
34	A	3	84	133-135
27	B	4	49	220-222
35 (PGP-4008)	B	3	52	157-159
36	B	2	55	189-191
37	B	1.5	45	195-197
38	B	2	53	160-162

[a]A: potassium *tert*-butoxide; B: sodium hydride. [b]Isolated yield.

Scheme 4.1. Synthesis of Substituted 1-Benzyl-dihydropyrrolquinolines

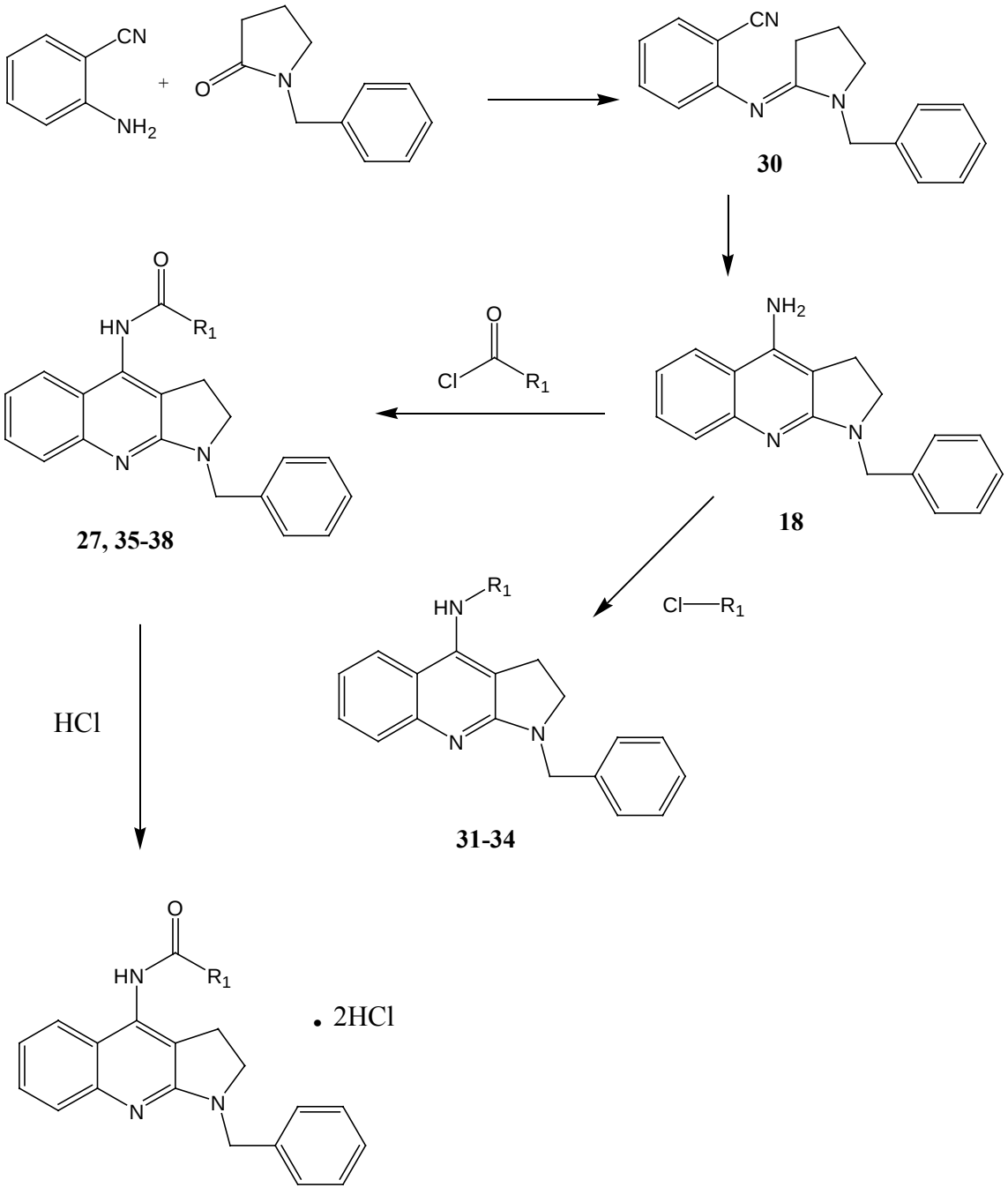
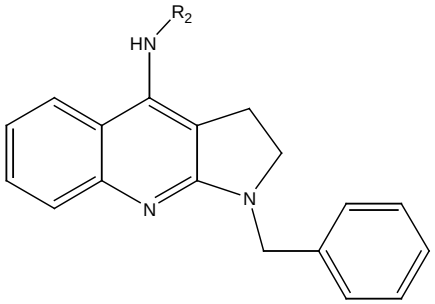
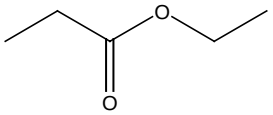
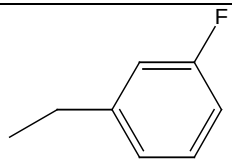
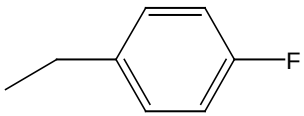
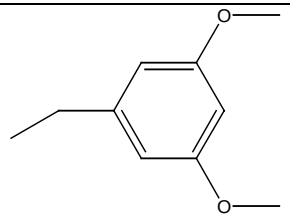
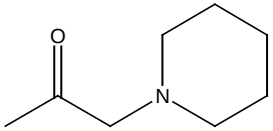
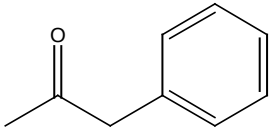
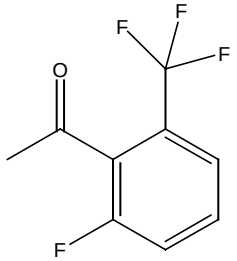
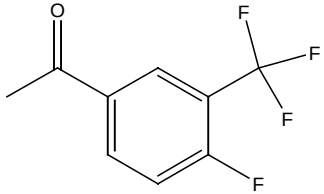
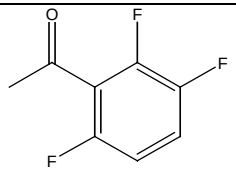


Table 4.3. Reversal of P-gp- and MRP1-Mediated MDR by pyrroloquinolines.



Compound	R_2	Toxicity IC_{50} (μM)	Antagonism at IC_{20} or less	
			P-gp	MRP
18	H	6.2	1.0	1.0
31		33	0.9	0.9
32		> 65	1.2	0.7
33		> 65	1.3	0.7

34		47	1.4 0.9
27		14	9.0 1.0
35 or PGP-4008		13	10.7 0.7
36		13	2.5 0.9
37		19	4.9 0.9
38		> 58	3.1 1.0

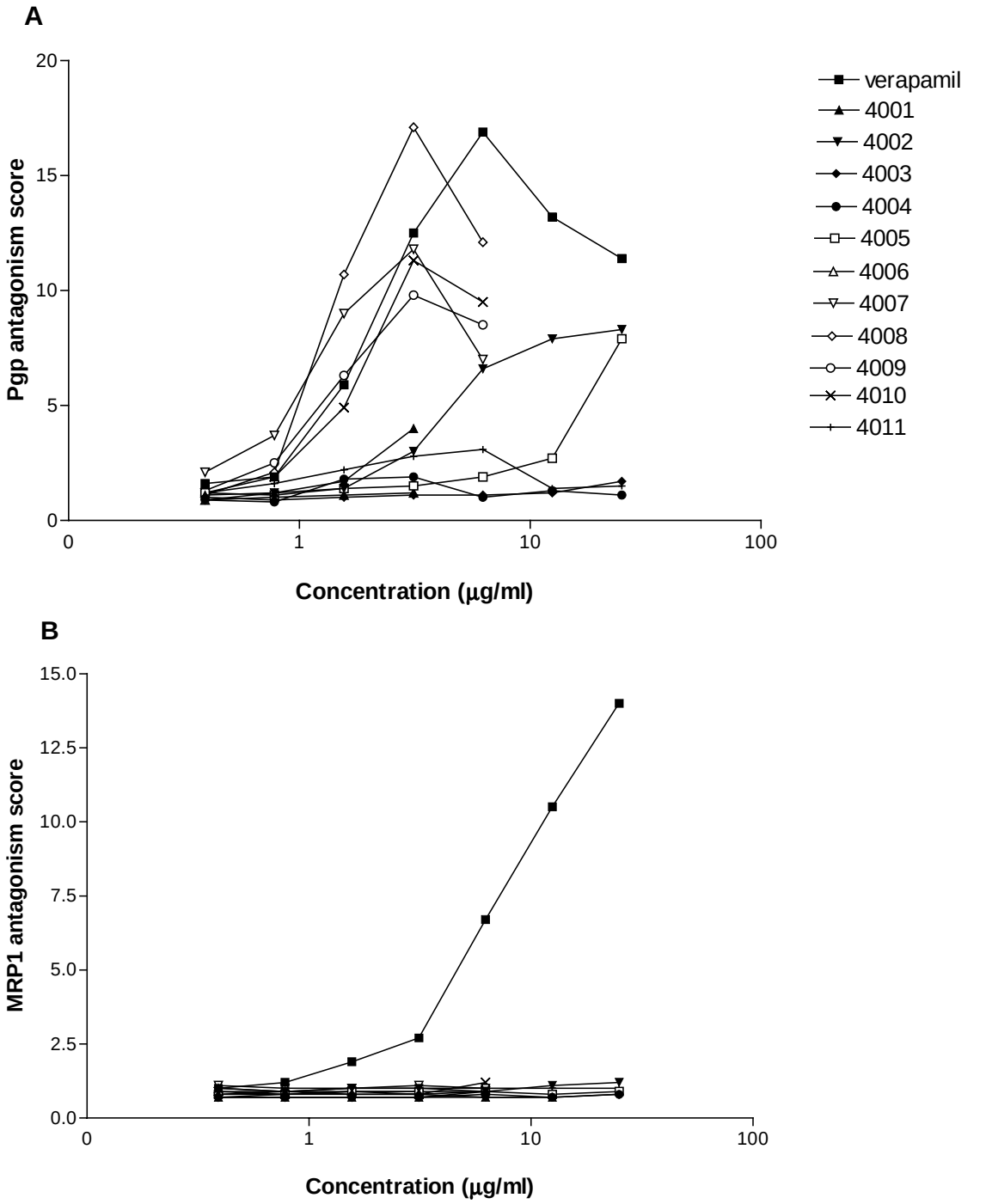


Figure 4.1. *In vitro* cytotoxicity assays of dihydropyrroloquinolines. (A) The P-gp Antagonism Score is calculated as the percentage of NCI/ADR cells surviving in the presence of 50 nM vinblastine / percentage of NCI/ADR cells surviving in the presence of vinblastine plus the indicated concentration of modulator. (B) The MRP1 Antagonism Score is calculated as the percentage of MCF-7/VP cells surviving in the presence of 1 nM vincristine / percentage of MCF-7/VP cells surviving in the presence of vincristine plus the indicated concentration of modulator.

Table 4.4. Cell line- and drug-specificity of the effects of PGP-4008 and Verapamil.

Cell Line	Drug	EtOH IC_{50}	Verapamil IC_{50}	RI	PGP-4008 IC_{50}	RI[a]
T24	Vinblastine (nM)	1.3 ± 0.3	0.5 ± 0.1	2.7	0.4 ± 0.1	3.8
	Taxol (nM)	4.7 ± 0.5	3.0 ± 0.4	1.6	4.0 ± 0.6	1.2
	Vincristine (nM)	4.8 ± 0.2	1.3 ± 0.2	3.7	2.2 ± 0.6	1.5
	Cisplatin (μM)	6.7 ± 1.1	7.0 ± 0.8	1.0	5.8 ± 1.2	1.1
	5-Fluorouracil (μM)	77 ± 20	48 ±6	1.6	29 ± 8	2.6
MCF-7	Vinblastine (nM)	0.4 ± 0.1	0.3 ± 0.1	1.2	0.2 ± 0	1.8
	Taxol (nM)	1.6 ± 0.3	1.4 ± 0.6	1.1	1.8 ± 0.1	0.9
	Vincristine (nM)	0.6 ± 0.1	0.1 ± 0.1	4.6	0.4 ± 0.1	0.9
	Cisplatin (μM)	16 ± 5	14 ± 4	1.1	19 ± 5	0.8
	5-Fluorouracil (μM)	49 ± 31	53 ± 20	0.9	30 ± 15	1.6
MCF7/VP (MRP1)	Vinblastine (nM)	0.6 ± 0.1	0.2 ± 0	2.4	0.4 ± 0.1	1.3
	Taxol (nM)	1.8 ± 0.4	1.6 ± 0.4	1.1	2.1 ± 0.4	0.8
	Vincristine (nM)	7.5 ± 1.1	0.5 ± 0.2	15.0	7.3 ± 0.9	1.0
	Cisplatin (μM)	8.5 ± 2.1	10.5 ± 3.2	0.8	11.3 ± 0.5	0.8
	5-Fluorouracil (μM)	11 ± 1	12 ± 2	0.9	16 ± 3	0.7
NCI/ADR (P-gp)	Vinblastine (nM)	110 ± 17	0.6 ± 0.1	173	3.1 ± 2.0	35
	Taxol (nM)	2020 ± 810	19 ± 5	106	29 ± 5	70
	Doxorubicin (μM)					
	Vincristine (nM)	183 ± 14	3.7 ± 0.8	50	10.5 ± 1.1	18
	Cisplatin (μM)	7.0 ± 0.9	6.3 ± 0.8	1.1	6.0 ± 0.5	1.2
	5-Fluorouracil (μM)	181 ± 84	210 ± 99	0.9	175 ± 74	1.0
P388/ADR (P-gp)	Vinblastine (nM)	28 ± 5	0.5 ± 0.1	61	0.5 ± 0.1	63
	Taxol (nM)	1650 ± 470	5.0 ± 1.4	330	8.2 ±2.9	202
	Doxorubicin (μM)	26.7 ± 10.6	0.03 ± 0.01	1000	0.13 ± 0.09	200
	Vincristine (nM)	150 ± 24	1.3 ± 0.5	112	5.0 ± 2.9	30
	Cisplatin (μM)	2.1 ± 0.8	1.1 ± 0.3	2.0	1.1 ± 0.4	1.9
	5-Fluorouracil (μM)	0.22 ± 0.04	0.30 ± 0.08	0.7	0.32 ± 0.08	0.7

[a] The Reversal Index (RI) is calculated as the ratio of the IC_{50} in the absence of modulator / the IC_{50} in the presence of modulator, so that larger values indicate increasing activity.

P-gp, NCI/ADR and P388/ADR cells, as indicated by the greater reversal index. In contrast, PGP-4008 did not strongly affect the toxicities of these drugs toward non-P-gp-overexpressing cell lines (T24 and MCF-7), nor do they affect the toxicities of the non-Pgp substrate drugs cisplatin and 5-fluorouracil toward any of the cell lines at doses sufficient to reverse P-gp-mediated MDR. PGP-4008 did not cause direct cytotoxicity to any of the cell lines at doses up to 250 μM, thereby providing a large therapeutic index. Furthermore, the selectivity of PGP-4008 is again illustrated in comparison to verapamil by the marked ability of verapamil to enhance the toxicity of vincristine toward MRP1-overexpressing MCF7/VP cells, whereas PGP-4008 has no effect on the toxicity of this drug.

Additional studies have shown that PGP-4008 increases the accumulation of [^{3}H]Taxol and [^{3}H]vinblastine by NCI/ADR cells without affecting the accumulation of these drugs by MCF-7 or MCF-7/VP cells (Figure 4.2). As with the *in vitro* cytotoxicity studies, the optimal effect of PGP-4008 was reached with doses below 2 μg/ml (5 μM). Therefore, the modulatory effects of PGP-4008 displayed in the *in vitro* cytotoxicity and drug accumulation assays are all consistent with selective antagonism of P-gp.

3. ***In vivo*** **evaluation of PGP-4008.** The most effective compound PGP-4008 was synthesized in larger quantities for *in vivo* studies. Initial experiments were hindered by the low solubility of the free base; however, conversion to the HCl salt markedly improved the solubility of PGP-4008. The therapeutic effects of PGP-4008-2HCl in reversing P-gp-mediated MDR were determined using an *in vivo* syngeneic solid tumor model consisting of JC murine mammary adenocarcinoma cells growing in Balb/c

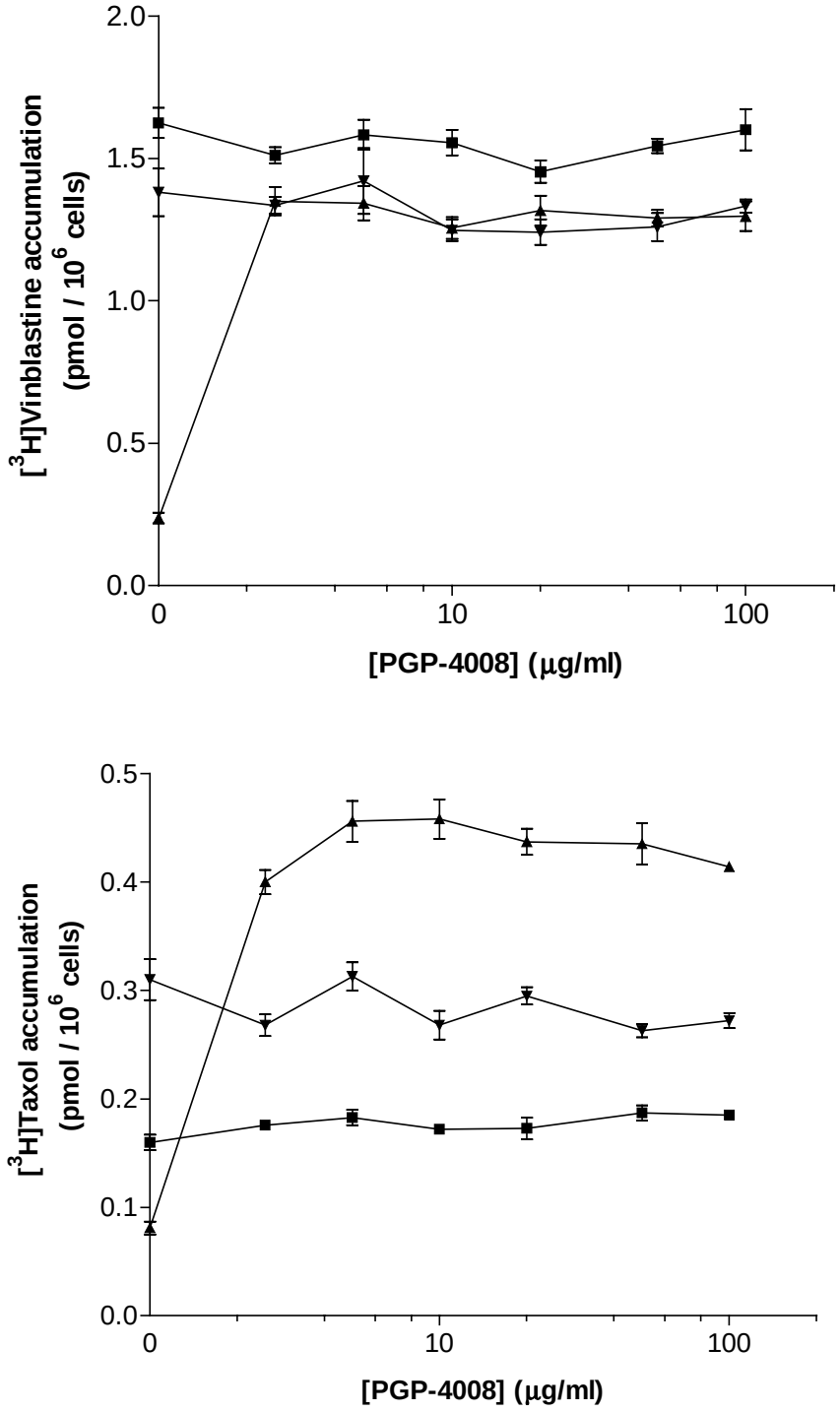


Figure 4.2. In vitro [^{3}H]-drug accumulation assay of PGP-4008. Intracellular accumulation of (A) [^{3}H]vinblastine and (B) [^{3}H]Taxol were performed using MCF-7 (■), NCI/ADR (▲), and MCF-7/VP (▼) cells and the indicated concentrations of PGP-4008 as described in the Materials and Methods section. Values represent the mean ± SD of triplicated samples in a representative experiment.

mice. The JC cells are resistant to a variety of anticancer drugs because of their high level of expression of P-gp. PGP-4008 effectively reverses the MDR phenotype of these cells (data not shown). In these xenograft studies, tumors are allowed to grow to approximately 400 mm^3 before the animals were treated with saline (control), 5 mg/kg doxorubicin, 100 mg/kg PGP-4008 or a combination of doxorubicin and PGP-4008. As shown in figure 4.3, the control group exhibited an increase in tumor volume of 500% by day 15. Animals treated with PGP-4008 alone or doxorubicin (a P-gp substrate) alone showed an increase in tumor size of 500% and 300%, respectively. In contrast, animals treated with the combination of doxorubicin and PGP-4008 experienced tumor growths of only 80% by day 15 ($P < 0.05$).

In similar studies, cyclosporin A was used to assess the toxicity profile of a non-specific modulator compared to the P-gp selective modulator, PGP-4008. Although cyclosporin A plus doxorubicin did inhibit tumor growth, there was a significant decrease (approximately 23%) in average animal weight that was not seen in mice treated with either agent alone (Table 4.5). This overall toxicity is consistent with the effects of cyclosporin A seen previously, and may be due to its lack of specificity for P-gp, alterations in the pharmacokinetics of doxorubicin or its immunosuppressive and nephrotoxic effects (Twentyman, 1988). However, there was no significant difference in average animal weight for mice receiving the combination of PGP-4008 plus doxorubicin. This supports the hypothesis that a P-gp specific MDR modulator, PGP-4008, will not contribute to the overall systemic toxicity when given in combination with a cytotoxic drug, yet can still reverse P-gp-mediated MDR.

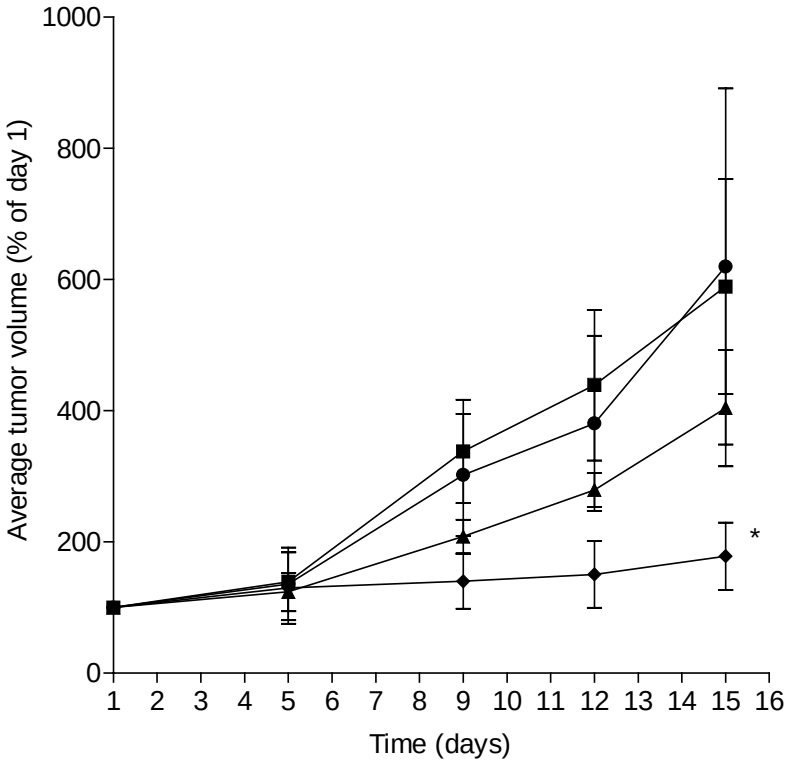


Figure 4.3. *In vivo* tumor growth inhibition by PGP-4008. Treatments of JC tumor bearing female Balb/c mice with PGP-4008 and/or doxorubicin were performed as described in "Materials and Methods." Tumor volumes for control (■), doxorubicin-treated (▲), PGP-4008-treated (●) or PGP-4008 plus doxorubicin-treated (♦) animals are shown as the mean ± SD (n = 4-5) of the percentage of the day 1 volume. *, $P < 0.05$.

Table 4.5. *In vivo* systemic toxicity comparison between a non-specific MDR modulator, cyclosporin A and a P-gp specific modulator, PGP-4008

	Day 1	Day 9	Day 15	Δ (Day 1 to 15)	% of Day 1
Control	20.0	20.9	22.2	2.2	11.0%
Doxorubicin	20.4	20.6	21.2	0.8	3.9%
Cyclosporin A	19.3	20.6	23.0	3.7	19.2%
Cyclosporin A + Doxorubicin	19.5	17.0	15.0	-4.5	-23.1%
Control	20.0	21.5	23.1	3.1	15.5%
Doxorubicin	21.4	20.6	20.2	-1.2	-5.6%
PGP-4008	20.9	21.0	22.1	1.2	5.7%
PGP-4008 + Doxorubicin	20.9	20.6	21.0	0.1	0.5%

Average mouse weights (g) from each group (n = 4-5).

Plasma pharmacokinetic studies of PGP-4008 were performed at doses similar to those used in the *in vivo* syngeneic solid tumor model. The plasma concentration profile of intraperitoneally administered PGP-4008 is shown in Figure 4.4, and the pharmacokinetic parameters are summarized in Table 4.6. Analysis of the concentration-time profile revealed that PGP-4008 best fits a two-compartment model, with a beta-phase terminal half-life of 1.2 hours. The highest concentration of PGP-4008 was observed at the earliest time point of 1 minute, indicating that PGP-4008 is rapidly absorbed into the systemic circulation from the intraperitoneal cavity. However, less than 1% of PGP-4008 remained in the plasma after 4 hours due to the rapid alpha-phase clearance. Intravenous studies showed that 60% of the dose of PGP-4008 passed the liver and intraperitoneal cavity. The area under the curve (AUC) corresponding to the remaining PGP-4008 was 17.6 μg·hr/mL and clearance rate from the plasma (Cl_p) was 80.4 mL/hr, corresponding to a large apparent volume of distribution (V_{dss} = 43 mL). The second compartment constitutes the majority of the volume of distribution of PGP-4008 (V_2 = 41 mL). Most important, however, is the fact that the maximum concentration in plasma (C_{max}, 197 μg/mL) is well above the *in vitro* effective dose of approximately 0.8 μg/ml. Furthermore, this effective concentration is maintained for 2 h indicating that PGP-4008 is rapidly absorbed into the blood stream at therapeutically significant levels.

Plasma distribution studies of a cytotoxic anticancer drug co-administered with PGP-4008 were performed at doses similar to those used in the *in vivo* tumor model (Figure 4.5). Additionally, this plasma distribution was measured over a time period (2 h) that correlated with the pharmacokinetic parameters of PGP-4008. The results indicate

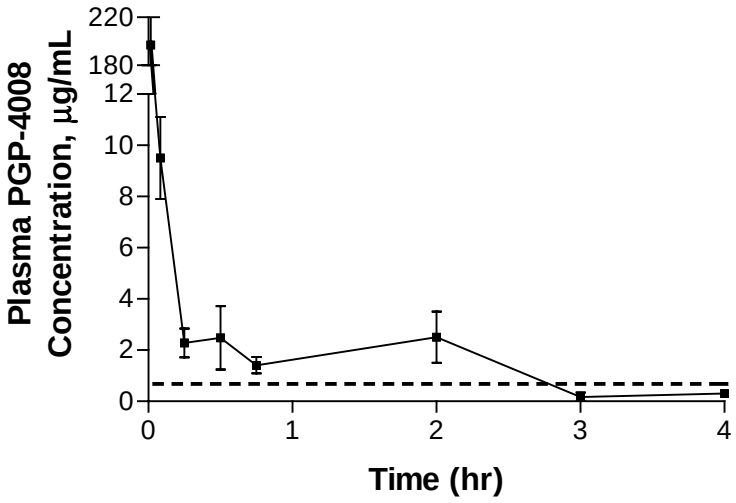


Figure 4.4. Pharmacokinetic plasma profile of PGP-4008. PGP-4008 (100 mg/kg) was administered intraperitoneally to mice and plasma samples were analyzed at the indicated times. Values represent the mean ± SD for three samples. Dashed line indicates effective *in vitro* dose, 0.8 µg/ml.

Table 4.6. Pharmacokinetic parameters of PGP-4008.

AUC	17.6 ± 1.4 mg×hr/mL
AUMC	9.4 ± 5.2 mg×hr2/mL
A	440 ± 6 mg/mL
B	2.25 ± 0.36 mg/mL
k_{α}	49 ± 0.9 hr-1
k_{β}	0.58 ± 0.19 hr-1
$t_{1/2\beta}$	1.20 ± 0.39 hr
k_{el}	35 ± 2.7 hr-1
V_{dss}	43 ± 15 mL
V_1	2.3 ± 0.3 mL
V_2	40.5 ± 7.4 mL
Cl_p	80.4 ± 6.4 mL/hr

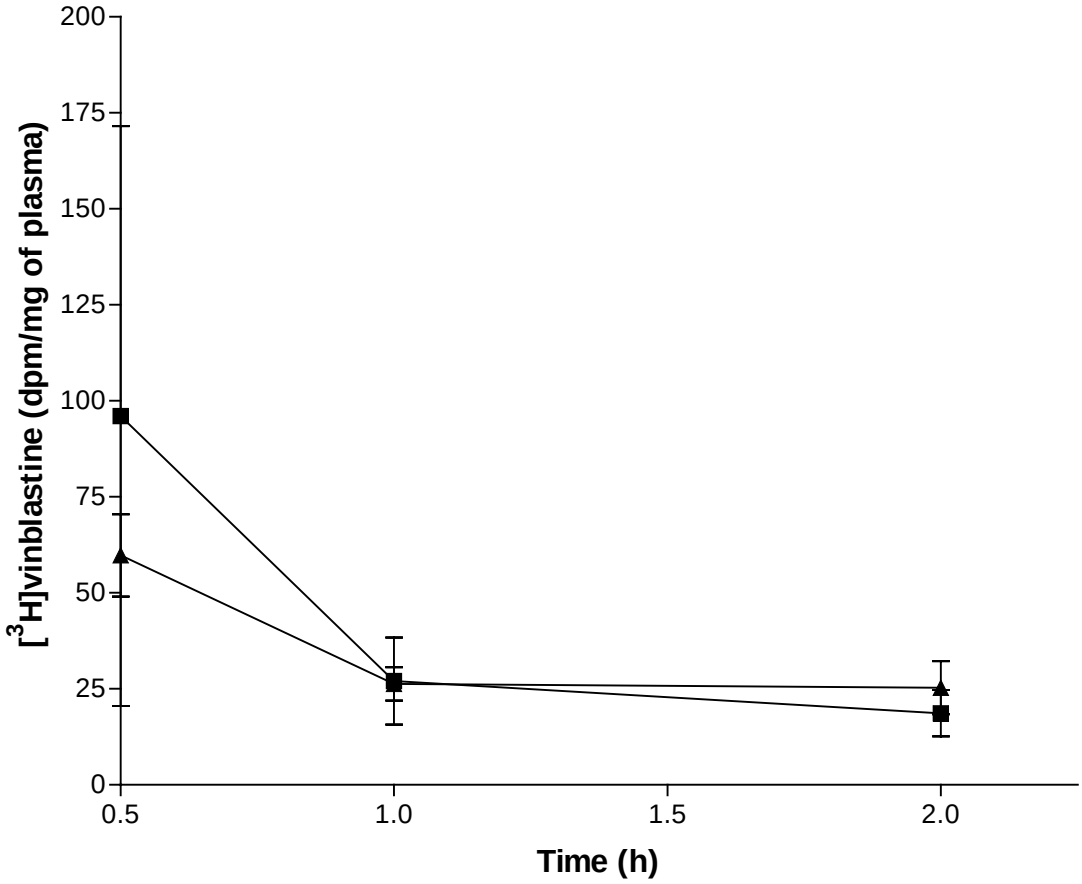


Figure 4.5. Plasma distribution of [^{3}H]vinblastine concomitantly administered with PGP-4008. Animal were treated with DMSO (■) or PGP-4008 (▲). PGP-4008 (100 mg/kg) was administered intraperitoneally to mice and samples were analyzed at the indicated times. Values represent the mean ± SD for three samples.

that the administration of PGP-4008 at a therapeutically relevant dose in mice did not alter the plasma distribution of the concomitantly administered cytotoxic drug. This contrasts with the altered pharmacokinetic properties commonly seen with non-specific MDR modulators.

D. Conclusions

We sought to develop MDR modulators that selectively antagonized P-gp to effectively potentiate the cytotoxicity of chemotherapeutic anticancer drugs towards resistant tumors. We hypothesized that P-gp selective MDR antagonists would be more clinically beneficial and lead to fewer adverse effects due to the more limited tissue distribution of P-gp compared with MRP1, and the evidence of its importance in clinical oncology. Screening libraries of structurally diverse compounds yielded several scaffolds on which to build new modulators. The pyrroloquinoline heterocycle was chosen for its ammenablility to synthesis and the availability of several substitution sites. Biological evaluation of dihydropyrroloquinoline analogs using *in vitro* assays demonstrated their highly selective modulation of P-gp with maximal activity demonstrated by PGP-4008. Furthermore, *in vivo* testing of PGP-4008 demonstrated its efficacy in the inhibition of a P-gp-mediated MDR in a solid tumor model, its rapid systemic absorption, and its lack of interaction with a concomitantly administered chemotherapeutic agent. These results indicate the effectiveness of PGP-4008 in the reversal of P-gp-mediated MDR and its potential for clinical utility.

CHAPTER 5

EVIDENCE OF A CELLULAR INFLUX MECHANISM FOR ACTINOMYCIN D USING THE P-GLYCOPROTEIN INHIBITOR, 2-BENZYLOXY-3-METHYL-QUINOXALINE

A. Introduction

Actinomycin D is a peptide-containing antibiotic isolated from *Streptomyces*. It is a potent antibiotic that has been used as an anticancer drug for the treatment of a variety of tumors, including rhabdomyosarcomas and Wilms' tumor in children as a component of VAC therapy (vincristine, actinomycin D, cyclophosphamide). In combination with primary surgery and radiotherapy, this therapeutic regimen has resulted in significant patient responses (Wadkins et al., 1996). Actinomycin D has also been shown to be an effective treatment for Ewing's tumor, Kaposi's sarcoma, soft tissue sarcomas, and advanced choriocarcinoma (Frei, 1974; Hardman and Limbird, 1996). Recently, actinomycin D has been shown to down-regulate the expression of Bcl-xL, an antiapoptotic protein, resulting in the sensitization of AIDS-Kaposi sarcoma and prostate tumor cells to Apo2L-mediated apoptosis (Mori et al., 1999; Ng et al., 2002). Actinomycin D is also the first small molecule drug capable of binding to single-stranded DNA (ssDNA) with high affinity and sequence specificity, resulting in inhibition of HIV-1 reverse transcriptase as well as other polymerases (Rill and Hecker, 1996).

Actinomycin D exerts its cytotoxic action by its binding to DNA (Sobell, 1973), through intercalation of the phenoxazone chromophore between guanine-cytosine base pairs with the benzenoid and quinonoid-linked cyclic pentapeptide lactone rings spanning two base pairs in opposite directions in the minor groove of the helix (Chen et al., 1996). This intercalation of actinomycin D blocks the transcription of DNA by RNA polymerase, and can produce double-stranded DNA breaks by acting as a topoisomerase II poison or through the generation of free radicals (Sobell and Jain, 1972; Ross and Bradley, 1981). In order for actinomycin D to exert its cytotoxic action upon a cell, it must enter the cell, pass into the nucleus, and bind to DNA. The biochemical mechanisms for these delivery events have never been fully characterized, leading to the current assumption that actinomycin D enters the cell through passive diffusion (Polet, 1975).

A series of substituted quinoxalinones was developed in our laboratory to inhibit P-gp and effectively reverse the multiple drug resistance (MDR) phenotype (Lawrence et al., 2001). MDR is a phenomenon in which tumor cells demonstrate intrinsic resistance or acquire resistance to a variety of structurally unrelated anticancer drugs. A major mechanism of MDR is through the overexpression of energy-dependent, unidirectional transmembrane efflux pumps such as P-gp, a member of the ATP-binding cassette (ABC) superfamily of transporters (Juliano and Ling, 1976). These transporters function by binding to the anticancer drugs within the cells and releasing them to the extracellular space using energy from the hydrolysis of ATP (Horio et al., 1988). The removal of the intracellular drug spares the tumor cells by not allowing the drug to interact with its intracellular target, such as actinomycin D binding to DNA (Kartner et al., 1983).

The substituted quinoxalinones increased the efficacy of a variety of anticancer drugs in P-gp expressing tumor cell lines (Lawrence et al., 2001). However, one compound, 2-benzyloxy-3-methyl-quinoxaline (termed BMQ), displayed an unexpected and surprising effect in increasing the accumulation of [^{3}H]actinomycin D in a variety of cell lines. This increased cellular accumulation is not due exclusively to inhibition of efflux by BMQ, leading us to further characterize the mechanism of actinomycin D influx into cells. Our results show that the accumulation of [^{3}H]actinomycin D and the augmentation by BMQ is energy-dependent, temperature-dependent, and saturable. These results implicate the existence of a BMQ-modulated membrane transporter responsible for the influx of actinomycin D into the cell.

B. Materials and Methods

Cell culture and cell lines. MCF-7 and NCI/ADR (Fairchild et al., 1987a) cells were obtained from the Division of Cancer Treatment of the National Cancer Institute. A-498, CV-1, Hep-G2, HT-29, MDA-MB-231, NIH/3T3, NRK, Panc-1, and T24 cells were obtained from American Type Cell Culture (Manassas, VA). A-498, CV-1, and Panc-1 cells were maintained in DMEM containing 10% FBS, 1 mM sodium pyruvate and 50 μg/ml gentamicin. NIH/3T3 cells were maintained in DMEM containing 10% calf serum, 1 mM sodium pyruvate and 50 μg/ml gentamicin. All remaining cells were maintained in RPMI 1640 (Life Technologies, Inc., Rockville, MD) with L-glutamine containing 10%

FBS and 50 μg/ml gentamicin. Cells were maintained at 37°C and 5% CO_2 in 100 mm x 20 polystyrene tissue culture dishes.

Cytotoxicity assay. Cells were seeded in 96-well tissue culture dishes at approximately 20% confluency and allowed to recover and attach for 24 h. Cells were then treated with varying concentrations of modulators and/or cytotoxic drugs for 48 h. The number of surviving cells remaining in each well was quantified with the sulforhodamine B (SRB) colorimetric assay (Skehan et al., 1990). Briefly, cells were washed with phosphate-buffered saline (PBS) and fixed to the plate with 10% trichloroacetic acid. The cells were then washed with water and stained with 0.4% SRB in 1% acetic acid. Cells were then rinsed with 1% acetic acid and 10 mM Tris base buffer was added to dissolve the SRB. The degree of absorbance was determined with a PerkinElmer HTS 7000 Plus BioAssay plate reader at a wavelength of 570 nm.

[^{3}H]Drug accumulation assay. Cells were seeded in 24-well tissue culture dishes at approximately 25% confluency and allowed to recover and grow to near confluency, approximately 3-4 days. Media was aspirated and replaced with serum-free media. Compounds were incubated for 30 min. at 37°C. Approximately 0.1 μCi of [^{3}H]Taxol, 75 Ci/mmol, [^{3}H]vinblastine, 7.3 Ci/mmol, [^{3}H]actinomycin D, 9 Ci/mmol, (Moravek Biochemicals, Brea, CA) or [^{3}H]daunomycin, 5 Ci/mmol (NEN, Boston, MA) was then added per well and the cultures were incubated for 60 min. at 37°C. Radioactive media was aspirated and cells were rapidly washed twice with ice-cold PBS. Intracellular [^{3}H]drug was solubilized with 1% sodium dodecyl sulfate (SDS) in water and quantified by liquid scintillation counting using UniverSol (ICN, Costa Mesa, CA).

[^{3}H]Drug efflux assay. Cells were seeded in 24-well tissue culture dishes at approximately 25% confluency and allowed to recover and grow to near confluency, approximately 3-4 days. Media was aspirated and replaced with serum-free media containing [^{3}H]actinomcyin D, 9 Ci/mmol, or [^{3}H]vinblastine, 7.3 Ci/mmol. Cells were incubated with 0.1 μCi of radioactive drug per well for 60 min at 37ºC. Media was aspirated, and the cells were washed with ice-cold PBS before the addition of serum-free media. Compounds were then added to the cells and incubated for 60 min. at 37°C. Media was aspirated and cells rapidly washed twice with PBS. Intracellular [^{3}H]drug was solubilized with 1% SDS and quantified by liquid scintillation counting. Total intracellular accumulation of [^{3}H]drug was measured by incubating cells for 60 min. at 37°C, washing cells with PBS and solubilizing cells with 1% SDS.

Energy-dependent drug accumulation assay. Cells were plated as previously described for the drug accumulation assay. Media was aspirated and replaced with either serum-free media or media containing 40 μg/mL antimycin A, 10 mM 2-deoxyglucose, and 2 mM sodium azide (ATP(-) media) and incubated for 30 min. at 37ºC. Compound BMQ, verapamil, or ethanol (vehicle control) were then added and incubated for 30 min. at 37ºC. Approximately 0.1 μCi of [^{3}H]actinomycin D, 9 Ci/mmol, was added to each well and incubated for 60 min at 37ºC. Media was aspirated and cells rapidly washed twice with ice-cold PBS. Intracellular [^{3}H]drug was solubilized with 1% SDS and quantified by liquid scintillation counting. ATP levels of the cells were measured with a bioluminescent somatic cell assay kit (Sigma, St. Louis, MO).

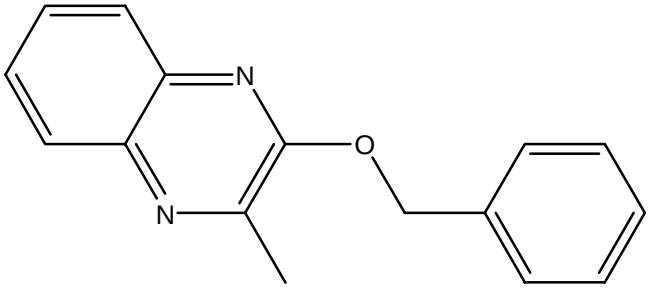
Temperature-dependent drug accumulation assay. Cells were plated as previously described for the drug accumulation assay. Media was aspirated and replaced with

serum-free media. Compound BMQ or ethanol was added to wells and incubated at 0ºC, 25 ºC, or 37ºC for 30 min. Approximately 0.1 μCi of [^{3}H]actinomycin D, 9 Ci/mmol, was then added per well and the cultures were incubated for 60 min. at the previous incubation temperature. Radioactive media was aspirated and cells were rapidly washed twice with ice-cold PBS. Intracellular [^{3}H]drug was solubilized with 1% SDS in water and quantified by liquid scintillation counting.

Drug accumulation saturation assay. Cells were plated as previously described for the drug accumulation assay. Media was aspirated and replaced with serum-free media. Cells were incubated with compound BMQ or ethanol for 30 min. at 37ºC. [^{3}H]Actinomycin D, 29 Ci/mmol, and actinomycin D (Sigma, St. Louis, MO) was then added to each well at varying concentrations and incubated for 60 min. at 37ºC. Radioactive media was aspirated and cells were rapidly washed twice with ice-cold PBS. Intracellular [^{3}H]drug was solubilized with 1% SDS in water and quantified by liquid scintillation counting.

C. Results

1. Effects of BMQ on actinomycin D cyctotoxicity and intracellular accumulation. We evaluated whether BMQ (Fig. 5.1) could reverse the classical P-gp-mediated MDR phenotype and potentiate the cytotoxicity of actinomycin D, a known P-gp substrate (Friche et al., 1989). In Fig. 5.2A, we treated NCI/ADR cells which



2-benzyloxy-3-methyl-quinoxaline (BMQ)

Figure 5.1. Chemical structure of 2-benzyloxy-3-methyl-quinoxaline (BMQ).

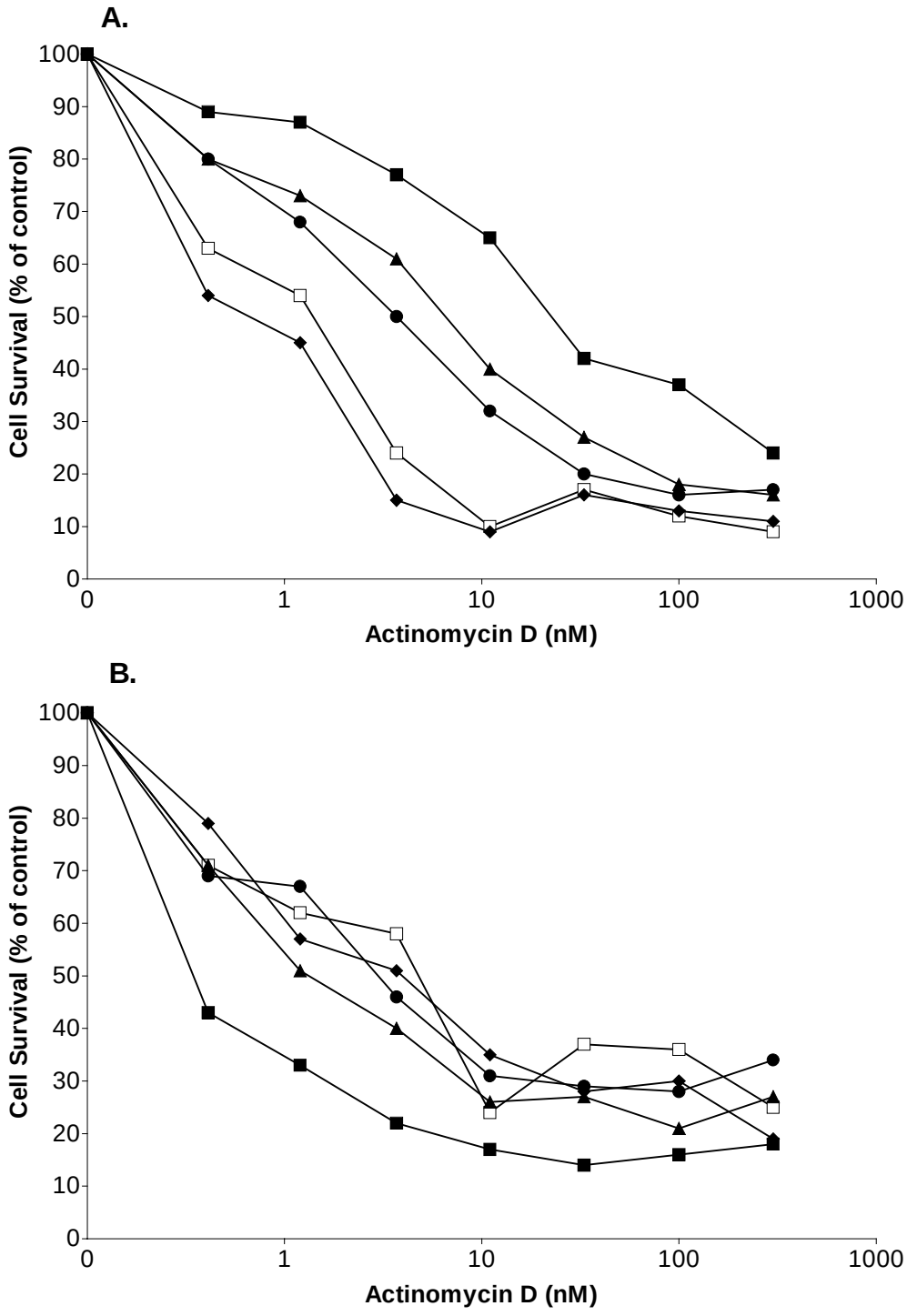


Figure 5.2. Effects of BMQ on potentiating the cytotoxicity of actinomycin D. Cell survival was determined by SRB colormetric assay in NCI/ADR cells (panel A) and MCF-7 cells (panel B). Cells were treated with increasing concentrations of actinomycin D in the presence of vehicle control, ethanol (■), BMQ at 2.5 μg/ml (▲), 5 μg/ml (●), or 10 μg/ml (♦), or verapamil at 10 μM (□). Data are mean ± S.D. calculated on experiments performed in triplicate.

markedly overexpress P-gp, with increasing concentrations of actinomycin D in combination with BMQ. The IC_{50} for actinomycin D shifted from approximately 25 nM to approximately 0.8 nM when the cells were treated with a final concentration of 40 μM of BMQ. This 30-fold potentiation of the cytotoxicity of actinomycin D by BMQ was very similar to the potentiation seen with verapamil, a known P-gp modulator. In Fig. 5.2B, MCF-7 cells, a drug sensitive breast adenocarcinoma, were treated with actinomycin D in combination with BMQ. BMQ did not increase the cytotoxicity of actinomycin D, which was expected since MCF-7 cells do not express the classical drug efflux transporters characteristic of MDR. These data indicate that BMQ, like the parental quinoxalinones, increase the cytotoxicity of actinomycin D by inhibiting the activity of P-gp.

Another well-established method for determining P-gp activity and the ability of modulators to inhibit P-gp is the measurement of the intracellular accumulation of a [^{3}H]drug that is efficiently effluxed by this transporter. When the P-gp overexpressing cells are treated with a P-gp modulator, there is a marked increase in the intracellular accumulation of the [^{3}H]drug. In Fig. 5.3, a variety of different cell types were treated with [^{3}H]actinomycin D in combination with BMQ. As expected, there was a large increase in the intracellular accumulation of [^{3}H]actinomycin D when the NCI/ADR cells were treated with BMQ. However, an unexpected observation was made in that BMQ also strongly increased the intracellular accumulation of [^{3}H]actinomycin D in drug-sensitive MCF-7 cells. Furthermore, BMQ displayed an ability to increase the intracellular accumulation of [^{3}H]actinomycin D in a wide variety of cell lines, representing a wide range of drug sensitivities as well as different species. BMQ

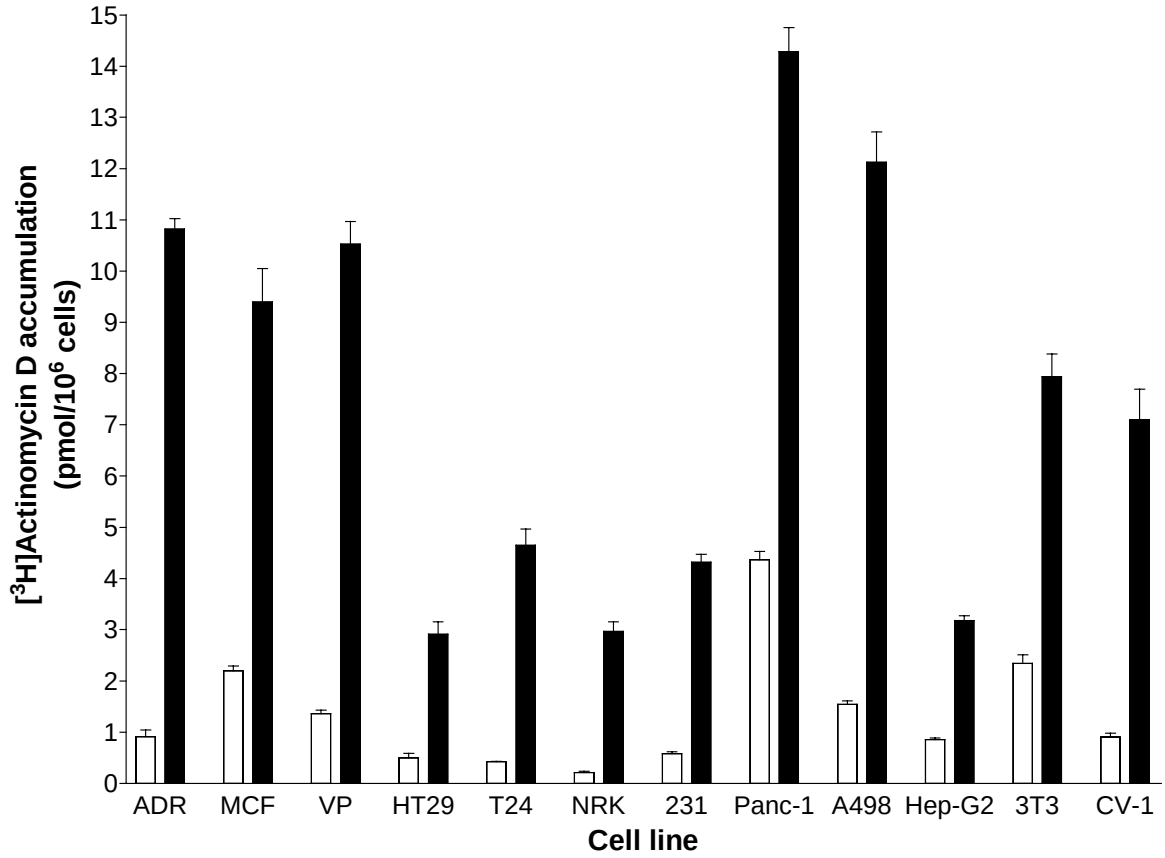


Figure 5.3. Effects of BMQ on facilitating the intracellular accumulation of [^{3}H]actinomycin D on a variety of cell lines. NCI/ADR (human breast adenocarcinoma with P-gp overexpression), MCF-7 (human breast adenocarcinoma), HT-29 (human colon adenocarcinoma), T24 (human bladder carcinoma), NRK (normal rat kidney), MDA-MB-231 (human breast adenocarcinoma), Panc-1 (human pancreatic carcinoma), A498 (human kidney carcinoma), Hep-G2 (human hepatocellular carcinoma), NIH/3T3 (mouse fibroblast), and CV-1 (monkey kidney) cells were incubated with ethanol (□) or BMQ (■) for 30 min before addition of [^{3}H]actinomycin D. Data are mean ± S.D. calculated on experiments performed in triplicate.

increased the accumulation of [^{3}H]actinomycin D from 3-fold to 11-fold in various cell lines. In marked contrast, BMQ did not affect the intracellular accumulation of [^{3}H]vinblastine, [^{3}H]Taxol or [^{3}H]daunomycin (all three are well-established P-gp substrates) in any cell line, except those that expressed P-gp (data not shown). The enhanced accumulation of [^{3}H]actinomycin D in the MCF-7 cell line was surprising since BMQ did not potentiate the cytotoxicity of actinomycin D.

We observed that BMQ increases the intracellular accumulation of [^{3}H]actinomycin D in cell lines that do not display the MDR phenotype. We next sought to determine whether BMQ increases the accumulation of [^{3}H]actinomycin D through the inhibition of the efflux of the drug or through the facilitation of the influx of the drug. In Fig. 5.4, MCF-7 cells were preloaded with [^{3}H]actinomycin D or [^{3}H]vinblastine before either BMQ or verapamil was added. The cells were then incubated for 60 min to determine if the modulators affect the efflux of the drug from the cells. BMQ did not significantly inhibit the efflux of either [^{3}H]actinomycin D or [^{3}H]vinblastine from MCF-7 cells. Efflux of [^{3}H]actinomycin D and [^{3}H]vinblastine from NCI/ADR cells was also determined. BMQ and verapamil significantly inhibited the efflux of both drugs from the cells, which is expected when the activity of P-gp is blocked. Therefore, the increased intracellular accumulation of [^{3}H]actinomycin D in MCF-7 cells by BMQ is not due to inhibition of efflux, but is due to the facilitation of influx of the drug.

2. Mechanism of actinomycin D influx and modulation by BMQ. The enhancement of actinomycin D uptake by BMQ is difficult to rationalize if uptake occurs

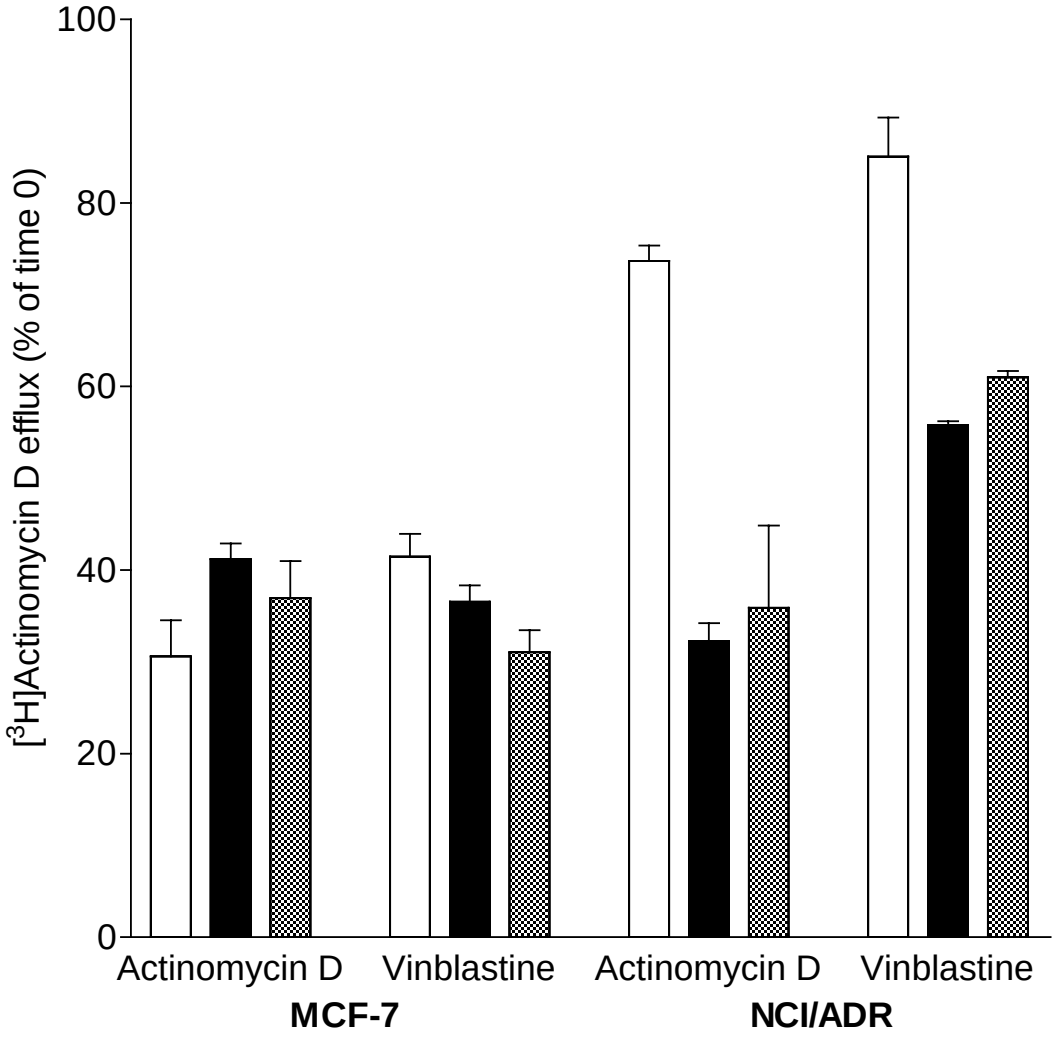


Figure 5.4. Effects of BMQ on efflux inhibition. MCF-7 and NCI/ADR cells were preloaded with [^{3}H]actinomycin D or [^{3}H]vinblastine, washed, then incubated with ethanol (□), 80 μM BMQ (■), or 20 μM verapamil (checkered). Intracellular accumulation of [^{3}H]drug was determined and compared to time 0 (total intracellular accumulation of [^{3}H]drug before addition of modulators). Data are mean ± S.D. calculated on experiments performed in triplicate.

by passive diffusion, as has been previously hypothesized. Therefore, we analyzed a variety of properties of actinomycin D uptake to determine if it is in fact carrier-mediated. Typical properties of carrier-mediated transport include saturability, temperature-dependence and energy-dependence.

To support the hypothesis of a carrier-mediated active transport mechanism for the influx of actinomycin D, we attempted to determine if the accumulation of actinomcyin D was able to reach saturation. In Fig. 5.5, MCF-7 cells were incubated with varying concentrations of actinomycin D, up to 1 mM. The initial accumulation of actinomycin D at lower concentrations follow zero-order kinetics, but as the concentration increases, the accumulation begins to plateau. Using non-linear regression with a one-site binding equation, a theoretical K_d and Bmax were determined. For the ethanol-treated cells, the K_d was 275 ± 7.3 μM with a Bmax of 19.2 ± 0.2 (R^2=0.9997). For the BMQ-treated cells, the K_d was 336 ± 53 μM with a Bmax of 23.1 ± 1.3 (R^2=0.99). The concentration of actinomycin D could not be increased higher than 1 mM due to solubility limits of the drug in an aqueous solution. Therefore, the data show that the accumulation of actinomycin D, as well as its facilitation by BMQ, is saturable.

Another hallmark of active transport is the inhibition of activity as the temperature decreases. In Fig. 5.6A, the accumulation of [^{3}H]actinomycin D with and without BMQ was measured at various temperatures in MCF-7 cells. At 4ºC, the accumulation of [^{3}H]actinomycin D is virtually abolished, and BMQ did not facilitate its intracellular accumulation. At 25ºC, the basal accumulation of [^{3}H]actinomycin D was also very low; however, BMQ increased the accumulation approximately 10-fold. The basal accumulation of [^{3}H]actinomycin D was optimal at 37ºC, and as demonstrated

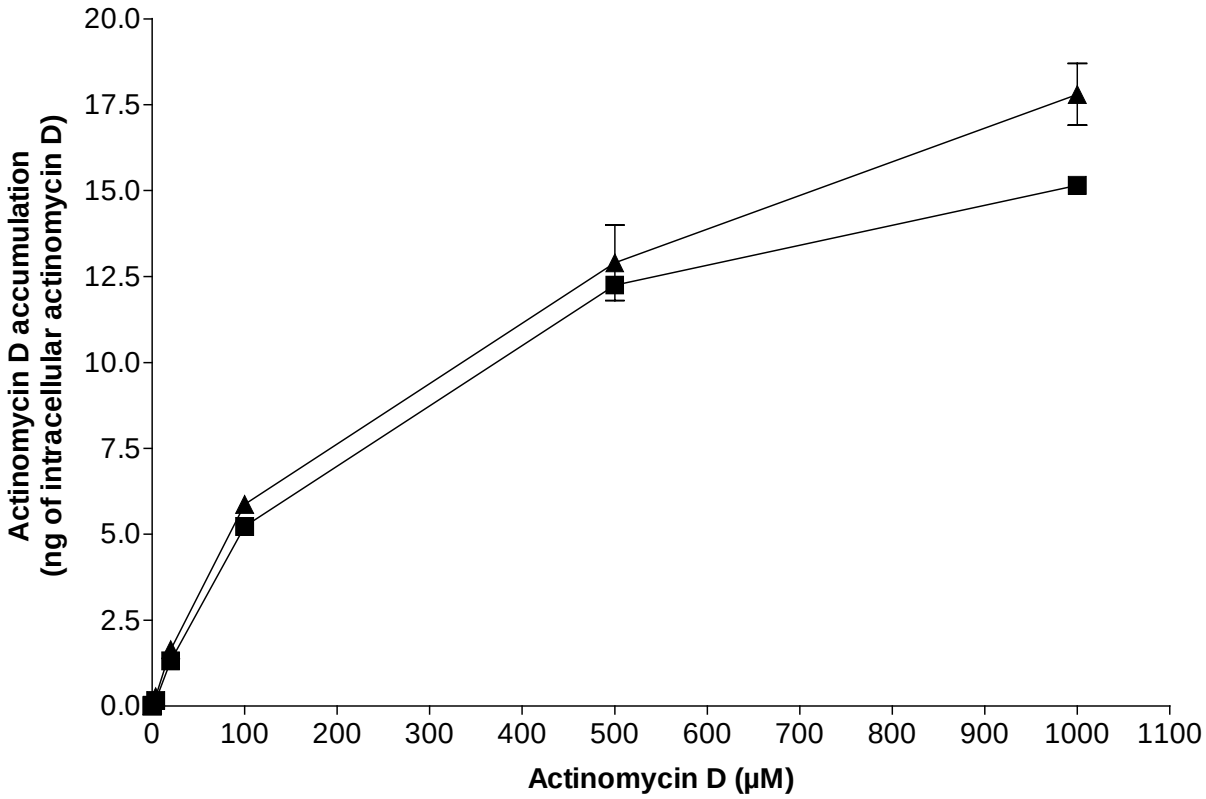


Figure 5.5. Saturation of [^{3}H]actinomycin D influx. MCF-7 cells were incubated with ethanol (■) or BMQ (▲) for 30 min before the addition of increasing concentrations of actinomycin D (4 nM to 1 mM) and further incubated for 60 min. Data are mean ± S.D. calculated on experiments performed in duplicate.

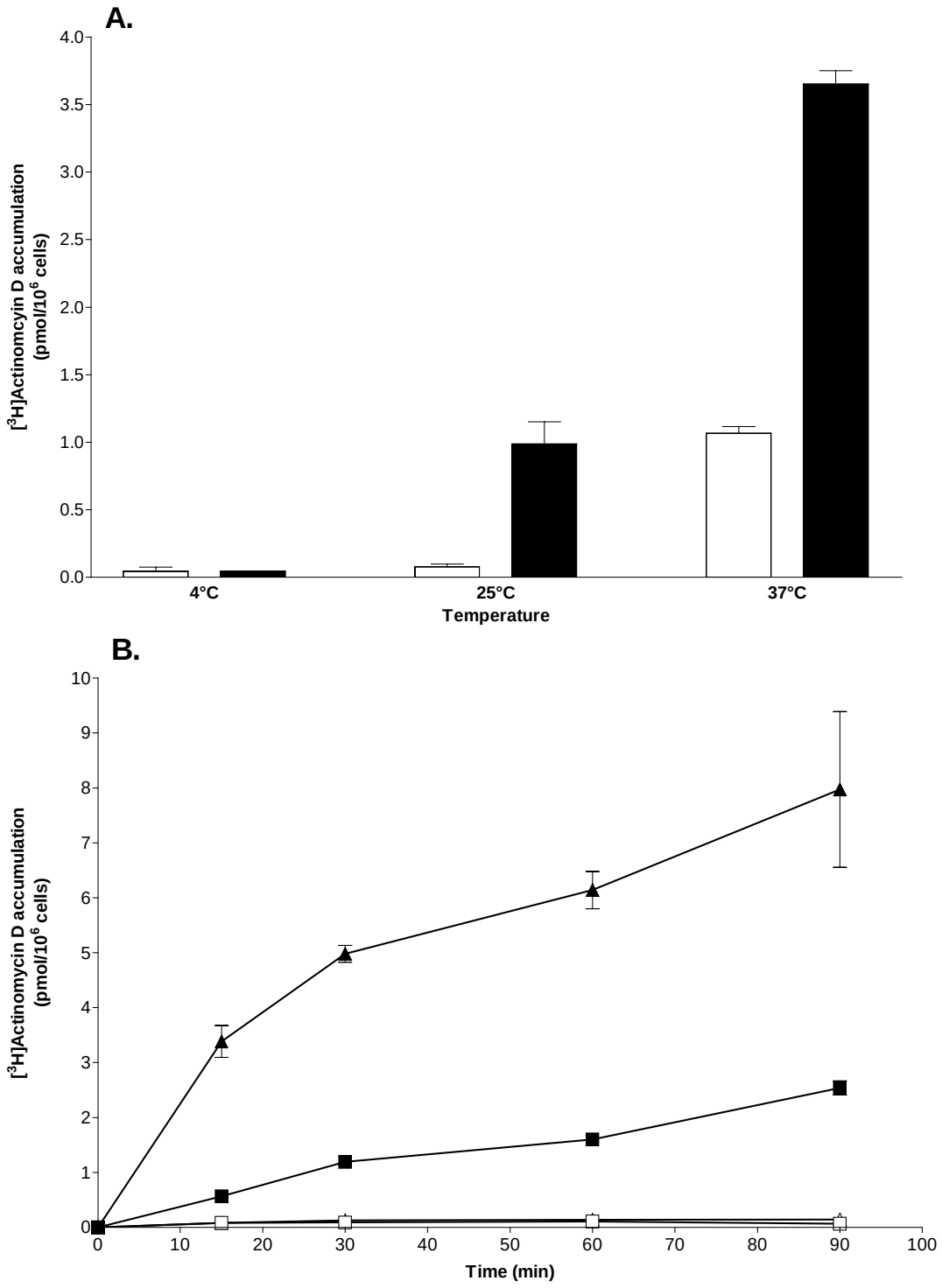


Figure 5.6. Temperature-dependent influx of [^{3}H]actinomycin D. MCF-7 cells were incubated with ethanol (□) or 80 μM BMQ (■) at 4ºC, 25ºC, or 37ºC (panel A). Intracellular accumulation in MCF-7 cells was measured at 4ºC with ethanol (□) or 80 μM BMQ (Δ) and 37ºC with ethanol (■) or 80 μM BMQ (▲) at 15, 30, 60 and 90 min after addition of [^{3}H]actinomycin D (panel B). Data are mean ± S.D. calculated on experiments performed in triplicate.

above, was significantly enhanced by the presence of BMQ. In Fig. 5.6B, the kinetics of the accumulation of [^{3}H]actinomycin D were measured in MCF-7 cells. There was no accumulation of [^{3}H]actinomycin D at 4ºC for at least 90 min, compared with the marked increase in accumulation at 37ºC and further 4-fold increase in the presence of BMQ. The data clearly shows that influx of [^{3}H]actinomycin D is temperature-dependent, which is consistent with a previous report (Polet, 1975; Goldman et al., 1981).

The energy-dependence for facilitation of actinomycin D influx by BMQ was assessed using antimycin A, 2-deoxyglucose, and sodium azide to deplete the MCF-7 and NCI/ADR cells of ATP. Antimycin A is an inhibitor of respiratory complex III of the electron transport system (Shen et al., 2000), and sodium azide inhibits electron transport at the level of cytochrome oxidase (Ronner et al., 2001). 2-deoxyglucose acts as a phosphate trap to remove free phosphate. Treatment with these agents reduced ATP levels in MCF-7 and NCI/ADR cells by approximately 75% (Figure 5.7 insert). This reduction of ATP in NCI/ADR cells was sufficient to enhance the basal accumulation of [^{3}H]actinomycin D and to block the ability of verapamil to increase [^{3}H]actinomycin D accumulation, indicating blockage of P-gp activity.

Treatment of MCF-7 or NCI/ADR cells with the ATP depleting media completely abolished the effects of BMQ on the intracellular accumulation of [^{3}H]actinomycin D. These data demonstrate that the facilitation of [^{3}H]actinomycin D influx by BMQ requires the presence of ATP.

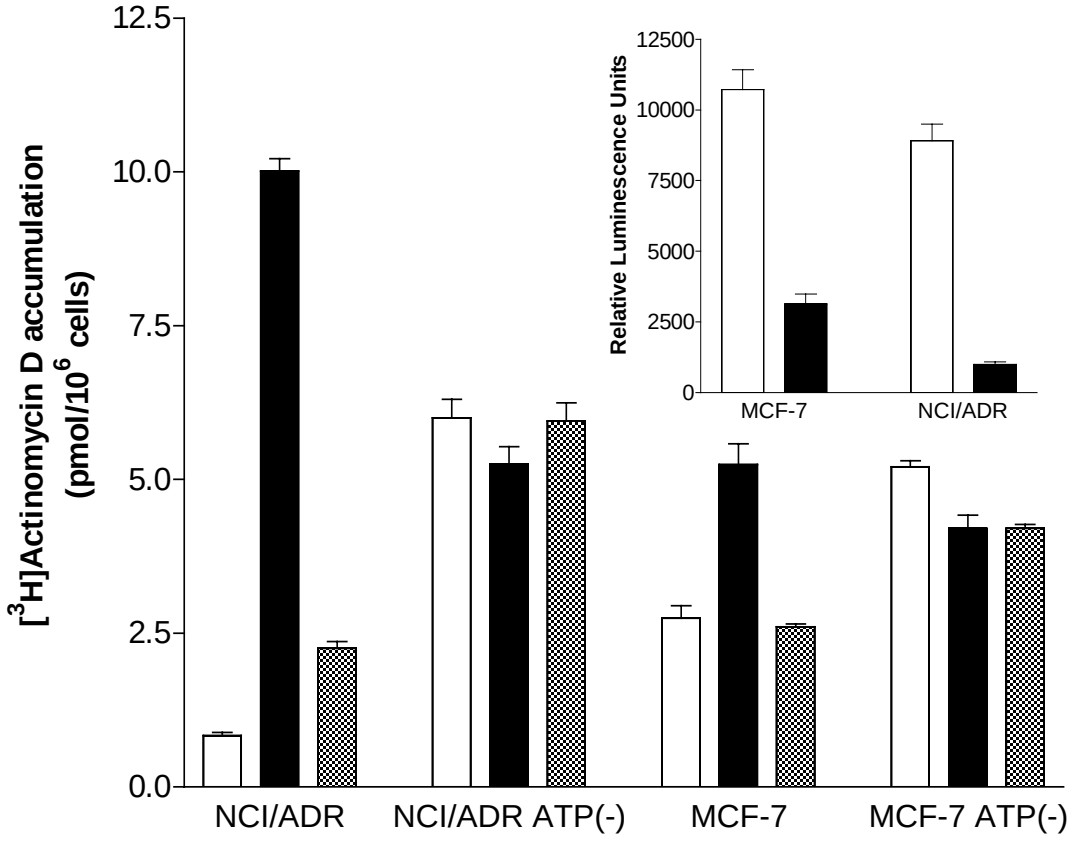


Figure 5.7. Energy-dependent influx of [^{3}H]actinomycin D. NCI/ADR and MCF-7 were incubated in normal media or media containing 40 μg/mL antimycin A, 10 mM 2-deoxyglucose, and 2 mM sodium azide (ATP(-) media). Cells were incubated with ethanol (□), 80 μM BMQ (■), or 10 μM verapamil (checkered). ATP levels (inset) within the cells treated with normal media (□) and ATP(-) media (■)were measured with a bioluminescent cell assay. Data are mean ± S.D. calculated on experiments performed in triplicate.

D. Discussion

2-Benzyloxy-3-methyl-quinoxaline, BMQ, was originally produced as a side-reaction product in the synthesis of a series of P-gp-inhibiting quinoxalinones (Lawrence et al., 2001). We have now shown that BMQ can effectively modulate P-gp and reverse the MDR phenotype *in vitro*. A surprising observation with BMQ is its marked ability to increase the intracellular accumulation of actinomycin D in both P-gp expressing cells and non-P-gp drug-sensitive cell lines. This increased accumulation of actinomycin D results from the facilitation of the influx of the drug, and this process is saturable, temperature-dependent, and energy-dependent. This evidence suggests that actinomycin D enters the cell through a carrier-mediated active transport mechanism.

Although BMQ increases the intracellular accumulation of [^{3}H]actinomycin D, it does not affect the cytotoxicity of actinomycin D toward MCF-7 cells. As further discussed below, one explanation could be that BMQ acts on a transporter located in the plasma membrane. Once inside the cell, actinomycin D must traverse the nuclear membrane to associate with DNA. Currently, there is no information available about the mechanism of actinomycin D entry into the nucleus, i.e. through nuclear pores, passive diffusion or carrier-mediated uptake. However, it seems unlikely that BMQ affects nuclear uptake since it does not potentiate the cytotoxicity of the drug. Alternatively, if there are no binding sites available on the DNA, there would be no increase in cytotoxicity. While it may seem unlikely that the low nanomolar concentrations used in the cytotoxicity and drug accumulation assays would saturate the DNA binding sites, the data indicate the accumulation of micromolar concentrations within the cells. Another

explanation for the lack of cytotoxicity potentiation with BMQ could be the high sequestration of both actinomycin D and doxorubicin in cells. Previous studies have shown that actinomycin D and doxorubicin can be sequestered and retained within cells, then slowly excreted in their active form (Polet, 1975; Robles et al., 1999). The exact mechanism of sequestration of actinomycin D is unknown, but this observation could explain the lack of correlation between increased intracellular accumulation of actinomycin D by BMQ and cytotoxicity.

The idea that actinomycin D enters the cell through active transport is in contrast to the currently accepted hypothesis that actinomycin D crosses the plasma membrane by passive diffusion (Polet, 1975; Goldman et al., 1981). It has been assumed that since actinomycin D is lipophilic, it passively diffuses through the plasma membrane and enters the cell. However, there are a number of lipophilic drugs such as melphalan (amino acid transporter) (Vistica et al., 1978) and methotrexate (folic acid transporter) (Henderson and Zevely, 1984) that have been shown to enter cells through active transport mechanisms. These transporters are important since decreases in their expression in tumor cells result in resistance to their substrate drugs. There has also been some previous evidence that supports the hypothesis that actinomycin D can be carried across the cellular membrane through an active process. It has been previously shown that the accumulation of actinomycin D is temperature-dependent, which is unexpected for a passive diffusional process (Bowen and Goldman, 1975; Polet, 1975; Goldman et al., 1981). The hypothesized mechanism for this temperature-dependence was that cooling impairs the diffusion permeability of the cell membrane (Polet, 1975), or that it may be related in part to the high thermal energy required to break actinomycin D hydrogen

bonds with water to permit penetration of the lipid membrane (Bowen and Goldman, 1975). However, another explanation could be that the decrease in temperature inhibits the activity of a transporter of actinomycin D.

Previous studies have reported that shortly after addition of actinomycin D to Ehrlich ascites tumor cells, there was a decline in the uptake rate to a lower constant velocity that cannot be attributed to a fall in the extracellular actinomycin D concentration (Goldman et al., 1981). It was hypothesized that the binding capacity of the cells is so immense that if measured over a longer interval, the points would linearize. However, while the possibility for an energy-dependent transporter of actinomycin D was recognized, there was no evidence at the time to support that hypothesis. In uptake studies performed in Ehrlich cells with concentrations of actinomycin D ranging from 0.02 to 14 μM, the linear influx kinetics support a zero-order (non-saturable) influx process (Bowen and Goldman, 1975). However, there remains the possibility of a low-affinity saturable transporter that could not be detected within these studies since the concentrations of actinomycin D used were too low. Data described herein indicate that this was the case, since the K_d for actinomycin D uptake is approximately 300 μM.

Hopefully, the study of the mechanism of actinomycin D transport will be of great value in the continued and future use of this drug in a clinical setting. Actinomycin D has been shown to be a vital drug in the treatment of human cancers including Wilms' tumor and rhabdosarcoma (Hardman and Limbird, 1996). Furthermore, with the discovery of its binding to ssDNA and subsequent inhibition of HIV-1 reverse transcriptase, this may provide a therapeutic regimen for combating HIV and other viruses that replicate through single-stranded DNA intermediates (Rill and Hecker, 1996). Drug discovery efforts in

designing novel antivirals that are selective for single-stranded DNA of the viral genome could lead to new drugs that cause less toxicity to the double-stranded DNA of the host.

In summary, we believe that the evidence of saturation, temperature-dependence and energy-dependence of the facilitated accumulation of [^{3}H]actinomycin D by BMQ indicates that a carrier-mediated mechanism is responsible for the transport of [^{3}H]actinomycin D across the cellular membrane. The use of BMQ has allowed the detection of this low-affinity transporter. Efforts are underway to identify this transporter since its characterization may lead to novel compounds to increase the efficacy and potency of actinomycin D and possibly analogs developed as anticancer or antiviral chemotherapy.

CHAPTER 6

CONCLUSIONS

Multidrug resistance (MDR) is a phenomenon by which tumor cells develop reduced sensitivity to anticancer drugs, which often leads to the failure of cancer chemotherapy. A prominent mechanism of MDR is the overexpression of the multidrug efflux pump, P-glycoprotein (P-gp), which decreases the intracellular accumulation of many anticancer drugs and lead to increased tumor growth. Because of the importance of P-gp in determining the success of chemotherapy, attempts have been made to develop compounds that act as antagonists of P-gp. These antagonists, often termed MDR modulators, generally lack anticancer activity. Their function is to block P-gp-mediated drug efflux so that a concomitantly administered anticancer drug can cause cell death in a drug-resistant tumor.

A significant limitation in the drug development of MDR modulators has been the lack of predictive *in vivo* tumor models. Commonly used *in vivo* models include the intraperitoneal growth of P388 mouse leukemia cells and their drug-resistant, P-gp overexpressing derivative, P388/ADR, which produce a sub-optimal system of directly administering both the modulator and cytotoxic drug directly into the cavity in which the cells have been implanted. This does not allow for the evaluation of the systemic distribution of drug and modulator. Other models involve xenograft transplantation of

resistant human epithelial tumor cells to form solid tumors in immunocompromised animals. This model lacks the ability to examine the effect of the immune system on the tumor, as well as any toxicity of the drug and modulator toward the immune system. While these models allow the evaluation of certain parameters of the test modulator, their disadvantages limit their overall utility. Therefore, we developed an *in vivo* tumor model with immunocompetent mice using the JC murine mammary adenocarcinoma cell line. This syngeneic P-gp-mediated MDR solid tumor model allows for the evaluation of the systemic distribution of drug and modulator as well as for the effects of the immune system on tumor growth and chemotherapy treatment.

It is likely that the lack of transporter-selectivity in early generation MDR modulators played a role in their ultimate failure. Therefore, we hypothesize that selectivity of individual MDR modulators to antagonize P-gp and increased potency will lead to less systemic toxicity and decreased alteration of the pharmacokinetics of the co-administered cytotoxic drugs. We identified a novel series of substituted pyrroloquinolines that selectively inhibits the function of P-glycoprotein (P-gp) without modulating multidrug resistance-related protein 1 (MRP1). One of the analogs, PGP-4008, showed the highest level of P-gp inhibition *in vitro.* Further evaluation of PGP-4008 *in vivo* demonstrated that it could inhibit tumor growth in the JC tumor model when given in combination with doxorubicin. PGP-4008 was rapidly absorbed into the bloodstream and achieved the *in vitro* effective dose up to 2 h after administration and did not alter the plasma distribution of concomitantly administered anticancer drugs. Signs of systemic toxicity were not seen with the P-gp selective modulator, PGP-4008, as

seen in comparison with a non-selective inhibitor, cyclosporin A, which demonstrates the importance of selectivity.

The development and characterization of P-gp-specific MDR modulators has led to an interesting observation between 2-benzyloxy-3-methyl-quinoxaline (BMQ) and the antitumor drug, actinomycin D. BMQ is able to markedly increase the intracellular accumulation of actinomycin D in variety of cell lines. The increased accumulation of actinomycin D by BMQ was not due exclusively to the inhibition of efflux from the cell. We demonstrated that actinomycin D uptake by cells is saturable, temperature-dependent and energy-dependent, suggesting an active transport mechanism. The idea that actinomycin D enters the cell through active transport is in contrast to the currently accepted hypothesis that actinomycin D crosses the plasma membrane by passive diffusion. However, studies described herein suggest that a membrane transporter is responsible for the passage of actinomycin D across the cellular membrane, and that BMQ can potentiate this influx mechanism.

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EDUCATION

1999-2003 The Pennsylvania State University, College of Medicine, Hershey, PA
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M.B.A.

1992-1996 University of California, Berkeley, CA
B.A. in Molecular and Cell Biology

AWARDS AND PROFESSIONAL AFFILIATIONS

2003 Young Investigator Award, Division of Drug Discovery, Development, and Regulatory Affairs, American Society for Pharmacology and Experimental Therapeutics (ASPET)

2003 Graduate Student Travel Award, ASPET

2002-Present Student Member, ASPET

2002-Present Member, Beta Gamma Sigma Honor Society for Schools of Business

2001-Present Associate Member, American Association for Cancer Research

PUBLICATIONS

Lee, B.D., French, K.J., Zhuang, Y. and Smith, C.D. Development of a syngeneic *in vivo* tumor model and its use in evaluating a novel P-glycoprotein modulator, PGP-4008. *Oncology Research.* 14(1), pp. 49-60, 2003.

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