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SHORT COMMUNICATION

MDR1 (*ABCB1*) G1199A (Ser400Asn) polymorphism alters transepithelial permeability and sensitivity to anticancer agents

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Abstract
Purpose P-glycoprotein (P-gp), encoded by *MDR1* (or *ABCB1*), is important in anticancer drug delivery and resistance. We evaluated alterations in P-gp-mediated transport of anticancer agents due to the *MDR1* G1199A polymorphism.
Methods Using stable recombinant epithelial cells expressing wild-type ($MDR1_{wt}$) or G1199A ($MDR1_{1199A}$), anticancer drug sensitivity and transepithelial permeability were evaluated.
Results The recombinant cells $MDR1_{wt}$ and $MDR1_{1199A}$ displayed comparable doxorubicin resistance. However, $MDR1_{1199A}$ cells displayed greater resistance to vinblastine, vincristine, paclitaxel, and VP-16 (11-, 2.9-, 1.9-, and 2.9-fold, respectively). Alterations in transepithelial permeability paralleled these changes. Efflux of doxorubicin was similar between $MDR1_{wt}$- and $MDR1_{1199A}$-expressing cells, while P-gp-mediated transport was greater for vinblastine and vincristine in $MDR1_{1199A}$ cells (2.9- and 2.0-fold, respectively).
Conclusions The occurrence and magnitude of the *MDR1* G1199A effect is drug specific. Overall, the *MDR1* G1199A polymorphism may impact anticancer efficacy through modulation of drug distribution and delivery to target tumor cells.

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Abbreviations

MDR1 or *ABCB1*	Multidrug resistance gene
P-gp	P-glycoprotein
ABC	ATP-binding cassette
BBB	Blood–brain-barrier
SNP	Single nucleotide polymorphism
$MDR1_{wt}$	Wild-type *MDR1*
$MDR1_{1199A}$	*MDR1* G1199A polymorphism
EC_{50}	Effective drug concentration necessary for 50% cell death
TEER	Transepithelial electrical resistance values
P_{app}	Apparent permeability
$P_{appA\rightarrow B}$	Apical-to-basolateral apparent permeability
$P_{appB\rightarrow A}$	Basolateral-to-apical apparent permeability

Introduction

The human multidrug resistance gene (*MDR1* or *ABCB1*) encodes a 170-kDa integral membrane protein, P-glycoprotein (P-gp), which mediates ATP-dependent substrate efflux. P-gp is a member of the ATP-binding cassette (ABC) superfamily of transporters that resides in the plasma membrane and functions as an efflux transporter for a wide variety of natural compounds and lipophilic xenobiotics [1]. Overexpression of P-gp in cancer cells causes a decrease in drug accumulation, thereby leading to the development of cellular resistance to anticancer agents.

P-gp also plays a role in the pharmacokinetics and disposition of anticancer drugs via its high degree of expression in intestinal mucosa, liver canalicular membrane, kidney proximal tubules, blood–brain-barrier (BBB), and placenta [1].

The sequence of *MDR1* gene is highly variable with more than 60 single nucleotide polymorphisms (SNPs) identified in the coding region, and displays substantial linkage disequilibrium. Genetic variation in *MDR1* has been shown to influence P-gp expression and function both in vitro and in vivo; however, results have been inconclusive and controversial [2]. Therefore, a systematic study designed to address the functional significance of *MDR1* genetic polymorphisms at the cellular and molecular levels is needed to define the impact on clinical pharmacokinetics. To accomplish this, we have developed a recombinant expression system in LLC-PK1 epithelial cells capable of expressing *MDR1* variants in a reproducible manner [3].

The model for this recombinant cell expression system was expression of the *MDR1* G1199A SNP, which results in a serine to asparagine transition at amino acid 400 (Ser400Asn) in a cytoplasmic domain of P-gp (allelic frequency ~5.5% in Caucasians) [3, 4]. Unlike other well recognized *MDR1* polymorphisms (e.g. C1236T, G2677T, and C3435T), G1199A is not in linkage disequilibrium with other SNPs. In our expression, we confirmed that similar levels of mRNA and protein are expressed in polarized LLC-PK1 cells expressing either wild-type *MDR1* ($MDR1_{wt}$) or the G1199A genetic polymorphism ($MDR1_{1199A}$) [3]. We also demonstrated that *MDR1* G1199A altered P-gp-mediated transport of a prototypic substrate, rhodamine-123, as well as HIV protease inhibitors [3, 5]. As a part of our initial study to validate these recombinant *MDR1* cells, we reported that *MDR1* G1199A altered cellular resistance to some anticancer agents in LLC-PK1 cells [3]. Our lab has also shown that *MDR1* G1199A alters chemoresistance in recombinant HEK cells [6]; and a recent study has shown *MDR1* G1199A may be predictive of progression-free survival in patients receiving paclitaxel chemotherapy [7]. Therefore, it appears that *MDR1* G1199A may be an important SNP in determining the clinical efficacy of anticancer agents.

In this report, we further evaluated the influence of *MDR1* G1199A on P-gp-mediated cellular resistance using an expanded panel of anticancer drugs, and compare the results to previous cytotoxic results. We also evaluated transepithelial permeability of anticancer agents in our recombinant system. Transepithelial permeability studies provide a tool to estimate the movement of a drug across a physiologic epithelial or endothelial membrane in the body; and therefore, predict distribution of drugs into target tissues in the body. Altered transepithelial permeability of anticancer agents due to *MDR1* polymorphisms may be particularly important at the BBB where P-gp presents a major hurdle to anticancer drug delivery into the central nervous system (CNS) for the treatment of brain tumors.

Materials and methods

Cell culture

LLC-PK1 control and recombinant *MDR1*-expressing cells were cultured in complete media consisting of RPMI medium 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal calf serum and 1% (v/v) antibiotic-antimycotic, and grown at 37°C in the presence of 5% CO_2. For transepithelial permeability studies, cells were cultured in Medium 199 (Invitrogen) supplemented with L-glutamine, 10% (v/v) fetal calf serum, and 1% (v/v) antibiotic-antimycotic.

Cytotoxic drug sensitivity assay

Sensitivity to cytotoxic anticancer agents was evaluated in LLC-PK1 control and *MDR1* recombinant cells as described previously [3]. Varying concentrations of doxorubicin, vinblastine, paclitaxel, VP-16 (Bedford Laboratories, Bedford, OH), and vincristine (Faulding, Paramus, NJ) were added to the cells in quadruplicate. Cells were incubated with the cytotoxic drugs for 3 days at 37°C. On day 2 of the incubation, 1 μCi of ^{3}H-thymidine (specific activity: 81.1 Ci/mM) was added to each well and incubated overnight. Cell death was estimated by the amount of ^{3}H-thymidine incorporation into DNA of treated cells compared to untreated. Cytotoxicity was measured as the effective drug concentration necessary for 50% cell death (EC_{50}) for each drug; EC_{50} values were estimated using a sigmoid E_{max} model on WinNonlin® software (Pharsight, Mountain View, CA).

Transepithelial permeability assay

LLC-PK1 control and recombinant *MDR1* cells were plated at a density of 2×10^6 cells/24 mm well on permeable supports (Transwell™; 3.0 μm membrane pore size; Corning) and grown for 4 days at 37°C in 5% CO_2; media was refreshed after 2 days. Fresh media was added to the cells 1 h before the initiation of the experiment, and transepithelial electrical resistance (TEER) values were measured with a Millicell®-ERS (Millipore, Billerica, MA). All transport assays were carried out at 37°C and corrections were made for the difference in volume in the apical and basolateral compartments. Transport of 10 μM doxorubicin (VHA PLUS®, Irving, TX) in Hanks' balanced salt solution (Invitrogen) supplemented with 25 mM D-glucose and 10 mM

HEPES (pH 7.2) was measured with a Gemini XS microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA) with SoftMax Pro® software (Molecular Devices): excitation at 490 nm and emission at 560 nm. Experiments with radiolabeled compounds ^{3}H-vinblastine and ^{3}H-vincristine (Moravek Biochemical, Brea, CA) were performed at concentrations of 5 μM (1 μCi per well) in Opti-MEM medium (Invitrogen). Inhibition of transport was performed at 1 μM GF120918 [N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide] kindly provided by GlaxoSmithKline (Research Triangle Park, NC). Aliquots of 50 μL were taken from apical and basal compartments up to 1 h for doxorubicin and up to 4 h for vinblastine and vincristine. Apparent permeability (P_{app}) was calculated in the apical-to-basolateral direction ($P_{appA\rightarrow B}$) and in the basolateral-to-apical direction ($P_{appB\rightarrow A}$) as described [5, 8]. Briefly, $P_{app} = [1/(A \times C_0)] \times (dQ/dt)$, where A is the surface area of permeable support, C_0 is the initial concentration in the donor compartment, and dQ/dt is the rate of transfer of compound into the acceptor compartment. The ratio of $P_{appB\rightarrow A}/P_{appA\rightarrow B}$ was estimated to evaluate P-gp-mediated directional efflux. Samples were run in quadruplicate.

Statistical analysis

Student's two-sided t test was used to evaluate differences between two sets of data. $p < 0.05$ were considered statistically significant.

Results

Sensitivity to cytotoxic agents

Overexpression of P-gp in cancer cells often mediates the development of cellular resistance to doxorubicin, vinblastine, vincristine, paclitaxel, and VP-16 in tumor cells. Therefore, cellular sensitivity to these anticancer agents was evaluated based on a dose–response response curve and estimation of EC_{50} values in LLC-PK1 control and $MDR1_{wt}$- and $MDR1_{1199A}$-expressing cells (Table 1). The genotypic effect of G1199A was not consistent across the drugs. As reported earlier, there was no difference in cellular sensitivity to doxorubicin was similar between $MDR1_{wt}$- and $MDR1_{1199A}$-expressing cells, indicating G1199A does not influence P-gp-mediated resistance to doxorubicin [3]. However, a significant effect was observed in cellular sensitivities to vinblastine, vincristine, paclitaxel, and VP-16. Cells expressing $MDR1_{1199A}$ displayed increased resistance to vinblastine (11-fold; $p < 0.0005$), vincristine (2.9-fold; $p < 0.05$), paclitaxel (1.9-fold; $p < 0.0005$), and VP-16 (> 2.9-fold; $p < 0.0005$) compared to cells expressing $MDR1_{wt}$. Therefore, the *MDR1* G1199A polymorphism may be important in enhancing cellular resistance to these drugs. Collectively, these data suggest that the influence of G1199A on drug resistance, as well as the magnitude of the effect, appears to be drug specific.

Table 1 Cellular sensitivities of LLC-PK1 control and recombinant *MDR1* cells to anticancer agents

LLC-PK1 cells	EC_{50} values (nm) ± SD[a,b]		
	Control	$MDR1_{wt}$	$MDR1_{1199A}$
Doxorubicin[c]	34.2 ± 3.1	155 ± 68*	120 ± 32**
Vinblastine[c]	0.66 ± 0.22	1.41 ± 0.51*	15.7 + 4.0**,††
Vincristine[c]	0.37 ± 0.12	1.18 ± 0.56*	3.41 ± 1.47*,†
Paclitaxel	2.71 ± 0.88	10.9 ± 0.4*	20.2 ± 2.0**,††
VP-16	787 ± 55	1,443 ± 306*	>4,153**,††

[a] Significant difference between control and *MDR1*-expressing cells (either wt or 1199A); * $p < 0.05$ and ** $p < 0.005$

[b] Significant difference between $MDR1_{1199A}$- and $MDR1_{wt}$-expressing cells; † $p < 0.05$ and †† $p < 0.0005$

[c] Data previously published in [3]

Transepithelial permeability

P-gp is a directional transporter expressed on the apical membrane of epithelial cells and effluxes substrates in a basolateral-to-apical direction. We evaluated differences in transepithelial permeability between $MDR1_{wt}$ and $MDR1_{1199A}$ cells by calculating the permeability ratio ($P_{appB\rightarrow A}/P_{appA\rightarrow B}$) that represents the net flux of a drug across an epithelial membrane. LLC-PK1 controls cells were expected to have low permeability ratios compared to cells expression *MDR1*. The potent P-gp inhibitor GF120918 was used to confirm the role of P-gp-mediated transport. TEER values, which indicate the integrity of the monolayers and formation of tight junctions, averaged 358 ± 47.8 Ω cm^2, which confirmed a tight monolayer before the initiation of experiments.

For these experiments, we chose three representative drugs: doxorubicin, vincristine, and vinblastine. Doxorubicin provides equivalent sensitivity between wild-type and G1199A cells, while vincristine and vinblastine exhibit a higher degree of drug resistance in cells expressing G1199A. Transepithelial permeability ratios for doxorubicin, vinblastine, and vincristine are shown in Table 2. For all three drugs, the estimates of the permeability ratios were derived from a linear range of transport across the epithelial cells, and therefore saturation of transport was not a factor. In the control cells, the permeability ratios were close to unity for doxorubicin, vinblastine, and vincristine, indicating no directional flux, and the ratios were unaffected by

Table 2 Apparent permeability ratios for anticancer agents in LLC-PK1 control and recombinant *MDR1* cells

LLC-PK1 cells	$P_{appB\rightarrow A}/P_{appA\rightarrow B} \pm SD^{a,b,c}$		
	Control	$MDR1_{wt}$	$MDR1_{1199A}$
Doxorubicin	0.76 ± 0.18	3.12 ± 1.03**	2.89 ± 0.78**
Doxorubicin + GF120918	1.40 ± 0.49	1.91 ± 0.63	1.67 ± 0.90
Vinblastine	1.21 ± 0.32	5.64 ± 0.58**	16.4 ± 1.3**,††
Vinblastine + GF120918	1.32 ± 0.16	2.68 ± 0.44‡	2.22 ± 0.27‡
Vincristine	1.34 ± 0.36	2.01 ± 0.20*	4.08 ± 0.68**,†
Vincristine + GF120918	1.01 ± 0.26	0.85 ± 0.11‡	1.08 ± 0.16‡

[a] Significant difference between control and *MDR1*-expressing cells (either wt or 1199A); * $p < 0.05$ and ** $p < 0.005$

[b] Significant difference between $MDR1_{1199A}$- and $MDR1_{wt}$-expressing cells; † $p < 0.005$ and †† $p < 0.0001$

[c] Significant difference in the absence and presence of GF120918; ‡ $p < 0.005$

GF120918 confirming that P-gp-mediated efflux was not observed in the control cells. Expression of either $MDR1_{wt}$ or $MDR1_{1199A}$ in epithelial cells increased permeability ratios for doxorubicin, vinblastine, and vincristine compared to control cells, indicating that P-gp expression enhanced drug efflux in the recombinant cell system.

We next compared the directional transepithelial permeability between $MDR1_{wt}$- and $MDR1_{1199A}$-expressing cells. We found no difference in the apparent permeability ratios for doxorubicin between $MDR1_{wt}$- and $MDR1_{1199A}$-expressing cells, indicating no genetic difference in the transport of doxorubicin. However, the apparent transepithelial permeability ratios were significantly greater in cells expressing $MDR1_{1199A}$ than $MDR1_{wt}$ for both vinblastine (2.9-fold; $p < 0.0001$) and vincristine (2.0-fold; $p < 0.005$), indicating cells expressing the *MDR1* G1199A polymorphism are more efficient at mediating net directional flux of vinblastine and vincristine across a polarized epithelial membrane. While we did observe a distinct genotypic effect, the effect appears to be drug specific, which is consistent with our observations in the cellular resistance experiments.

In the presence of the P-gp inhibitor, GF120918, permeability ratios were dramatically reduced in both $MDR1_{wt}$- and $MDR1_{1199A}$-expressing cells for vinblastine and vincristine. While doxorubicin permeability ratios were reduced, the results were not significant. Genotypic differences observed in vinblastine and vincristine permeability ratios were also eliminated by GF120918 in $MDR1_{wt}$- and $MDR1_{1199A}$-expressing cells. Although the permeability ratios for vinblastine were not reduced to that of the control cells in the presence of GF120918, the values were similar between cells expressing $MDR1_{wt}$ and $MDR1_{1199A}$. These data suggest that G1199A enhances the transepithelial permeability of vinblastine and vincristine, but not doxorubicin, by increasing P-gp-mediated transport.

Discussion

The development of multidrug resistance in cancer cells and tumors is a major obstacle in the chemotherapy. Many anticancer agents used clinically are substrates of P-gp, and expression of P-gp in cancer cells and tumors is a primary cause of drug resistance. Interindividual variation in P-gp activity, due to *MDR1* polymorphisms, may influence distribution and delivery of anticancer agents to target cells and tissues. We have previously shown that recombinant cells expressing either $MDR1_{wt}$ or $MDR1_{1199A}$ provide a highly reliable and consistent method with which to evaluate functional changes in P-gp due to *MDR1* pharmacogenetics [3, 5]. This system has now been used to study the phenotypic impacts of the G1199A polymorphism in transepithelial permeability and cellular sensitivities to anticancer agents. Our system provides the ability to predict how drugs will move across cellular barriers in the body, including the intestine, liver, kidney, and BBB. Since the majority of chemotherapeutic agents are administered intravenously, distribution across the BBB may be of considerable interest with respect to drug delivery, efficacy, and toxicity.

Many anticancer agents have limited distribution into the CNS, partly due to poor penetration across BBB and efficient removal by P-gp-mediated efflux transport [9–13]. However, some antineoplastic agents have had some success in treating brain tumors in vivo. Vincristine is currently included in many treatment programs for children with tumors arising within or outside the CNS [14, 15]. Doxorubicin and vincristine are also both often used in the treatment of brain tumors and neuroblastomas [16–18]. Alterations in P-gp activity, due to *MDR1* polymorphisms, may influence anticancer agent distribution into the CNS as well as cellular sensitivity to cytotoxic drugs.

Utilizing our recombinant cell expression system, we evaluated the genotypic differences in cellular sensitivities to doxorubicin, vinblastine, vincristine, paclitaxel, and VP-16 in epithelial cells expressing either $MDR1_{wt}$ or $MDR1_{1199A}$. While drug resistance to doxorubicin was similar between $MDR1_{wt}$- and $MDR1_{1199A}$-expressing cells, $MDR1_{1199A}$-expressing cells displayed significantly enhanced resistance to vinblastine, vincristine, paclitaxel, and VP-16. This enhanced resistance in the $MDR1_{1199A}$-expressing cells would indicate that P-gp-mediated transport is enhanced in these cells. The resistance may be due to decreased intracellular accumulation, and therefore, reduced cytotoxicity. Differential sensitivity to anticancer agents may impact the efficacy of chemotherapy, and these results may be useful

in providing alternate choices of drugs to overcome chemoresistance in cancer patients with the G1199A polymorphism.

We also assessed the role of *MDR1* G1199A in P-gp-dependent directional transepithelial permeability in our recombinant expression system and found that changes in transepithelial permeability correspond to the alterations observed in cellular sensitivities. Transepithelial permeability of doxorubicin was not affected by expression of either $MDR1_{wt}$ or $MDR1_{1199A}$ indicating that the G1199A polymorphism does not impact doxorubicin disposition. Doxorubicin degradation in cardiomyocytes has been shown to be minimal even up to 4 h of incubation; therefore, we do not expect that extensive metabolism masked the effect of differential transport by the G1199A SNP [19]. However, $MDR1_{1199A}$-expressing cells exhibited significantly higher apparent transepithelial permeability ratios for both vinblastine and vincristine compared to cells expressing $MDR1_{wt}$. Therefore, our studies suggest that the effect of the *MDR1* G1199A polymorphism appears to be drug specific. LLC-PK1 cells express low levels of relevant drug-metabolizing enzymes; therefore, we do not expect that differential drug metabolism is playing a role in the drug specific pharmacogenomic effect we observed [20]. Since the P-gp-mediated transport of vinblastine and vincristine is more efficient in cells expressing $MDR1_{1199A}$, expression of G1199A may impact the permeability of anticancer agents across cell barriers in the body including the intestine, liver, kidney, and BBB. Since vinblastine and vincristine are administered intravenously, the effect of G1199A polymorphism may be most pronounced in their delivery and distribution across the BBB and in their clearance from the body. Since the *MDR1* G1199A polymorphism enhances transepithelial permeability of vinblastine and vincristine, we would expect that systemic clearance may be increased via enhanced biliary or renal secretion. Enhanced efflux at the BBB would contribute to further exclusion of distribution of vinblastine and vincristine into the brain. Therefore, our results indicate that distribution of vinblastine and vincristine into the CNS may be limited in cancer patients expressing G1199A, and that these patients may be more susceptible to the development of drug resistance.

The P-gp-mediated efflux of anticancer agents in $MDR1_{wt}$- and $MDR1_{1199A}$-expressing cells was almost completely inhibited in the presence of GF120918; that is, to the level of the control cells. These data indicate that the asymmetric effect of G1199A expression on transepithelial permeability of anticancer agents fully reflects the alteration in P-gp function. While GF120918 may also inhibit the breast cancer resistance protein (BCRP), there was no effect of GF120918 inhibition on the transepithelial permeability of anticancer agents in the control cells, indicating that BCRP does not play a role in our data interpretation.

In summary, we found that the *MDR1* G1199A polymorphism alters cellular sensitivity and transepithelial permeability of anticancer agents in this recombinant cell system. However, the occurrence and magnitude of a G1199A effect appears to be drug specific. *MDR1* G1199A displayed no effect on the disposition of doxorubicin, but significantly enhanced drug resistance and transepithelial permeability of vinblastine and vincristine as well as enhanced drug resistance to paclitaxel and VP-16. The *MDR1* G1199A polymorphism may impact cancer chemotherapeutic efficacy by influencing delivery of anticancer agents to target cells and distribution into the CNS. Further phenotype–genotype association studies are needed to confirm these predictions.

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