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Evaluation of the MDR-MDCK cell line as a permeability screen for the blood–brain barrier

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Abstract

The objectives of this study were to (1) characterize MDR-MDCK monolayers as an in vitro model to predict brain uptake potential; (2) examine the ability of MDR-MDCK monolayers to identify the brain uptake potential of compounds that interact with P-glycoprotein (P-gp). The study measured the bi-directional transport of 28 compounds across MDR-MDCK monolayers. The brain uptake of a subset of the compounds was determined in the rat brain perfusion model. Drug concentrations were analyzed by LC–MS–MS. CNS-positive drugs exhibited absorptive permeability coefficients (Papp, A–B) values ranging from 3.4×10^{-6} to 20.2×10^{-6} cm/s; whereas CNS-negative drugs showed Papp (A–B) ranging from 0.03×10^{-6} to 0.83×10^{-6} cm/s. Inhibition of P-gp by cyclosporin A (CsA) significantly reduced secretory flux of compounds known to be P-pg substrates, but only enhanced the absorptive flux of compounds with high efflux ratio (>100). In vitro results were confirmed by brain perfusion studies on selected compounds. MDR-MDCK monolayers can be used to classify compounds into CNS-positive or CNS-negative based on the permeability coefficients (Papp, A–B). Under our experimental conditions, compounds with Papp (A–B) $>3 \times 10^{-6}$ cm/s have high brain uptake potential; compounds with Papp (A–B) $< 1 \times 10^{-6}$ cm/s are unable to penetrate the blood–brain barrier (BBB); the brain uptake of compounds with Papp (A–B) $< 1 \times 10^{-6}$ cm/s and a P-gp-mediated efflux ratio of >100 may be enhanced by inhibiting P-gp.

Keywords: MDR-MDCK; Blood–brain barrier; Cell model; Permeability

1. Introduction

The primary interfaces between the central nervous system (CNS) and the systemic circulation are the blood–brain barrier (BBB), formed by the cerebral capillary endothelium, and the blood–cerebrospinal fluid (CSF) barrier, formed by the choroid plexus epithe-

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lium (Bendayan et al., 2002). The barrier at each site consists of a continuous single layer of cells joined by tight junctions that provide a highly regulated environment for the brain to function normally. In addition, expression of efflux proteins (i.e., P-glycoprotein (P-gp)) predominately localized in the luminal membranes of endothelial cells further restricts the entry of lipophilic compounds into the CNS (Lee et al., 2001). From a drug delivery point of view, the most desirable outcome is to enhance the BBB penetration by CNS drugs for maximal intended pharmacological effects, while reducing the BBB penetration by non-CNS drugs for minimal adverse neurological effects. Therefore, it is important to select drug candidates possessing desirable brain uptake potential.

Progress has been made in both in vivo and in vitro methodologies to study drug transport across the BBB. In vivo BBB experiments can provide valuable insight on drug permeation across the BBB, such as regional drug distribution, but these studies are laborious and require complicated analytical methods to measure plasma or brain drug concentrations. Thus, various in vitro systems have been used for studying BBB permeability. Brain microvessel endothelial cells, either primary cultures or cell lines, have been investigated from various mammalian species (Gumbleton and Audus, 2001).

There are several drawbacks associated with the use of primary cell culture systems for BBB permeability screening, including (a) time and labor associated with cell isolation and (b) batch-to-batch variability (Gumbleton and Audus, 2001). Brain endothelial cell lines provide a stable source with high yield and homogeneity. Thus, immortalized cell lines from bovine (Otis et al., 2001), rat (Yang and Aschner, 2003) and porcine (Franke et al., 2000) origin have been established. These cell lines form intercellular junctions, retain BBB enzymatic activities, and express BBB-specific cell surface markers. However, an important limitation of these cell lines is their low transendothelial electrical resistance (TEER). For example the measured TEER of bovine brain microvascular endothelial cells (BBMEC) has been reported in the range of 160–200 $\Omega\,cm^2$ (Raub et al., 1992). In contrast, the TEER of tight junctions in vivo is 2000 $\Omega\,cm^2$ or above (Crone and Olesen, 1982). Low TEER reflects loose intercellular junctions and is associated with high, passive paracellular diffusion. The permeability coefficients for sucrose were 10×10^{-6} cm/s in BBMEC (Raub et al., 1992) and 214×10^{-6} cm/s in the RBE4 cell line, derived from rat brain microvascular endothelial cells (Rist et al., 1997). Both systems lack sufficient paracellular restriction to be representative models of the BBB. Caco-2 cells are widely employed to predict oral absorption potential (Hidalgo, 2001); however, Caco-2 cell permeability did not predict in vivo BBB transport (Lundquist et al., 2002; Faassen et al., 2003).

In addition, Di et al. (2003) put forward parallel artificial membranes coated with porcine brain lipids (PAMPA-BBB) as a high throughput permeability assay for BBB. Also, computational models to predict BBB permeability of compounds were developed (Luco, 1999; Liu et al., 2004). However, lack of ability to integrate the effects of protein carriers in these processes greatly limits their use as reliable in vitro models for BBB permeability. This is particularly true if we consider that a high percentage of compounds interact with membrane transporters (Dresser et al., 2001).

Although the utility of MDR-MDCK, MDCK-II transfected with the human MDR1 gene, as a BBB permeability model has been proposed (Gumbleton and Audus, 2001), its potential utility cannot be assumed but needs to be demonstrated experimentally. First, MDR-MDCK cells are not endothelial but epithelial cells. Second, they were derived from dog and not human. Third, they are from kidney and not from brain capillaries. Mahar Doan et al. (2002) carried out pioneering work to investigate the utility of MDR-MDCK as a BBB model: passive permeability and efflux ratio of 93 drugs. A pitfall of this study was that they used the classification of drugs into CNS-indicated or non-CNS-indicated as the reference to judge the accuracy of the MDR-MDCK model to predict CNS uptake. In other words, CNS-indicated drugs were assumed to penetrate the BBB and non-CNS-indicated drugs were assumed not to penetrate the brain. However, these assumptions might be dangerous. Non-CNS-indicated drugs might have substantial brain uptake potential. CNS-indicated drugs might need only minimal brain uptake. CNS uptake potential may be better categorized based on in vivo brain uptake rather than therapeutic indications. Thus, in the present study a set of compounds were selected based on data obtained from in vivo or in situ rat or mice brain uptake experiments (Table 1).

Table 1
Information of compounds used in the study

Compounds	MW	Brain uptake classification[a]
Amitriptyline	277	CNS+ (Uhr et al., 2000)[b]
Antipyrine	188	CNS+ (Lundquist et al., 2002)[b]
Caffeine	194	CNS+ (Lundquist et al., 2002)[b]
Carbamazepine	236	CNS+ (Scheyer et al., 1994)[c]
Chlorpromazine	319	CNS+ (Mamo et al., 2004)[b]
Clonidine	230	CNS+ (Barber and Reid, 1982)[d]
Desipramine	267	CNS+ (Absorption Systems)[b]
Doxylamine	270	CNS+ (Absorption Systems)[b]
Fluoxetine	309	CNS+ (Liu et al., 2004)[b]
Guanfacine	246	CNS+ (Barber and Reid, 1982)[d]
Indomethacin	358	CNS+ (Shadid et al., 1998)[b]
Atenolol	266	CNS− (Street et al., 1979)[e]
Bretylium tosylate	414	CNS− (Schreiber and Sokolovsky, 1985)[f]
Cefuroxime	424	CNS− (Kourtopoulos et al., 1985)[f]
Chlorothiazide	296	CNS− (Absorption Systems)[b]
Furosemide	331	CNS− (Absorption Systems)[b]
Lincomycin	407	CNS− (Absorption System)[b]
Methotrexate	454	CNS− (Murakami et al., 2000)[b]
Ranitidine	351	CNS− (Canena et al., 1998)[d]
Sulfasalazine	398	CNS− (Absorption Systems)[b]
Digoxin	781	CNS− (Murakami et al., 2000)[b]
Dipyridamole	505	CNS− (Bisserbe et al., 1986)[g]
Etoposide	589	CNS− (Ogasawara et al., 1993)[d]
Labetalol	365	CNS− (McNeil and Louis, 1984)[f]
Loperamide	514	CNS− (Schinkel et al., 1996)[d]
Quinidine	324	CNS− (Murakami et al., 2000)[b]
Rhodamine 123	381	CNS− (Castro et al., 1992)[d]
Vinblastine	909	CNS− (Murakami et al., 2000)[b]

[a] In vivo study. Methods of CNS penetration.
[b] Brain perfusion study.
[c] Microdialysis.
[d] Brain to plasma ratio.
[e] Brain uptake index.
[f] Competition binding.
[g] Autoradiographic recording.

The objective of this study was to assess MDR-MDCK cells as a potential in vitro brain-uptake screening model. The study was carried out in two parts. In the first part, the bi-directional permeability coefficients of marketed drugs that are known to penetrate the BBB were measured to determine whether there was a qualitative correlation between MDR-MDCK cell permeability and in vivo CNS activities. In the second part, the role of P-gp as a determinant of the BBB penetration potential classification of drugs that interact with P-gp was examined.

2. Materials and methods

2.1. Materials

All tested compounds were purchased from Sigma (St. Louis, MO, USA). The selection of compounds was based on available clinical indications, i.e., information that supports brain penetration, such as clinical site of effect and in vivo animal experiments or known absence of brain penetration (Table 1). MDR-MDCK cells were obtained from NIH (Bethesda, MD, USA) and maintained in Minimum essential Eagle's medium containing 2 mM L-glutamine, 20 mM sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, 10% fetal bovine serum and supplemented with 0.2 nM colchicine (maintaining selective pressure). For transport experiments, cells with passage numbers 24–33 were seeded at a density of 60,000 cells/cm^2 on rat-tail type I collagen-coated polycarbonate membranes in 12-well transwell plates (Costar, MA, USA).

2.2. Cell culture and transport studies

The experiments were performed on the 8th day after seeding. The permeability assay buffer was Hank's balanced salt solution containing 10 mM HEPES and 15 mM glucose at pH 7.4. The cells were dosed on the apical side (A–B) or basolateral side (B–A) and incubated at 37 °C with 5% CO_2 and 90% relative humidity. The test compounds were prepared to final concentrations of 50 μM for CNS-negative compounds and 5 μM for CNS-positive compounds, as listed in Tables 2 and 3. The concentrations chosen were dependent on both the permeation of the compounds and the detection sensitivity of analytical methods. Among CNS-negative compounds, a number of them are P-gp substrates, which may saturate P-gp and then mask the effects of this efflux protein at high concentration (i.e., 50 μM); thus, the final concentration of 5 μM was used for the well-known P-gp substrates in the present study, as listed in Table 3 (bold font). In the P-gp inhibition study, the cells were pre-incubated with 10 μM cyclosporin A (CsA), a known P-gp inhibitor, for 30 min and the same concentration of CsA was present in the assay buffer throughout the experiments. Each determination was performed in quadruplicate. Samples were taken every 30 min for 2 h.

Table 2
Permeability and efflux ratio of CNS-positive compounds

Compounds	Dosing concentration (μM)	Papp ($\times 10^{-6}$ cm/s)		Efflux ratio (Papp (B–A)/Papp (A–B))
		A–B	B–A	
Amitriptyline	5	3.57 ± 0.14	9.88 ± 0.88	2.8
Antipyrine	5	19.35 ± 3.43	17.72 ± 1.64	0.9
Caffeine	5	20.19 ± 2.99	22.21 ± 0.88	1.1
Carbamazepine	5	19.62 ± 1.86	27.35 ± 1.47	1.4
Chlorpromazine	5	6.18 ± 0.71	16.27 ± 1.82	2.6
Clonidine	5	15.87 ± 1.39	13.61 ± 1.72	0.9
Desipramine[a]	5	5.56 ± 1.01	37.41 ± 3.25	6.7
Doxylamine[b]	5	3.39 ± 0.14	37.42 ± 1.16	11
Fluoxetine	5	8.08 ± 0.81	21.74 ± 0.78	2.7
Guanfacine	50	5.04 ± 0.36	15.48 ± 1.23	3.1
Indomethacin	5	13.67 ± 1.52	53.44 ± 4.45	3.9

Papp-values were expressed as mean ± S.D.; $n = 4$ for each determination.
[a] P-gp substrate (Watt et al., 1990).
[b] No reference so far has indicated that doxylamine is a P-gp substrate.

The apparent permeability coefficient, Papp, was calculated as follows:

$$\text{Papp} = \frac{\mathrm{d}Qr/\mathrm{d}t}{A \times C_0}$$

$\mathrm{d}Qr/\mathrm{d}t$ is the cumulative amount in the receiver compartment versus time; A the area of the cell monolayer; C_0 the initial concentration of the dosing solution.

2.3. Brain perfusion procedures

Adult male Sprague–Dawley rats were obtained from Hilltop Labs (Scottdale, PA, USA) and were housed in a temperature-controlled animal facility of West Chester University, PA, USA. All procedures were approved by the Institutional Animal Care and Use Committee of West Chester University, PA, USA, and were conducted in accor-

Table 3
Permeability and efflux ratio of CNS-negative compounds

Compounds	Dosing concentration (μM)	Papp ($\times 10^{-6}$ cm/s)		Efflux ratio (Papp (B–A)/Papp (A–B))
		A–B	B–A	
Atenolol	50	0.13 ± 0.03	0.25 ± 0.004	1.9
Bretylium tosylate	50	0.22 ± 0.06	1.13 ± 0.82	5.1
Cefuroxime	50	0.10 ± 0.02	0.13 ± 0.01	1.2
Chlorothiazide	50	0.25 ± 0.06	0.22 ± 0.01	0.9
Furosemide	50	0.028 ± 0.014	0.035 ± 0.003	1.2
Lincomycin	50	0.10 ± 0.04	0.32 ± 0.01	3.2
Methotrexate	50	0.11 ± 0.02	0.20 ± 0.02	1.8
Ranitidine	50	0.16 ± 0.04	0.87 ± 0.04	5.4
Sulfasalazine	50	0.11 ± 0.07	0.14 ± 0.05	1.3
Digoxin	5	0.28 ± 0.12	9.75 ± 0.46	35
Dipyridamole	5	0.30 ± 0.09	38.67 ± 1.60	127
Etoposide	5	0.13 ± 0.001	1.80 ± 0.26	14
Labetalol	5	0.30 ± 0.07	18.19 ± 1.44	61
Loperamide	5	0.18 ± 0.03	42.64 ± 1.15	237
Quinidine	5	0.34 ± 0.06	114.82 ± 19.36	338
Rhodamine 123	5	0.83 ± 0.04	7.63 ± 0.94	9
Vinblastine	5	0.40 ± 0.17	10.77 ± 0.71	27

Papp-values were expressed as mean ± S.D.; $n = 4$ for each determination. Compounds in bold font are well-known P-gp substrates.

dance with accepted standards for laboratory animal care. Brain uptake in rats was determined using a previously reported method (Smith et al., 1985; Smith, 1996). Briefly, perfusion was performed using the single time-point method. The perfusion fluid (perfusate) containing the test compound(s) was infused into rats ($N = 4$–11) via the left external carotid artery at a constant rate by an infusion pump. Perfusion flow rate was set to completely take over fluid flow to the brain at normal physiologic pressure (80–120 mmHg). The duration of the perfusion was 30 s, which was commonly used by other investigators (Smith, 1996; Killian et al., 2000; Murakami et al., 2000). Immediately following the perfusion, the brain vasculature was perfused for an additional 30 s with drug-free perfusate to remove residual drug. The pump was turned off, the brain was removed from the skull, and the left cerebral hemisphere was immediately excised and weighed. The whole left hemisphere was analyzed for drug concentration.

2.4. Sample analyses

All compounds, except for Rhodamine 123, were analyzed by LC–MS–MS. All mass spectrometry was conducted on a Sciex API2000 or API4000 triple quadrupole mass spectrometer in the MRM mode using a turbo ionspray interface. Mass spectrometer response for each analyte was individually optimized. The LC equipment consisted of two Perkin–Elmer PE200 micropumps and a PE200 autosampler. Chromatography was conducted in the reverse phase mode using a 30 mm × 2.1 mm i.d. BDS Hypersil C_{18} column. The mobile phase buffer was 25 mM ammonium hydroxide adjusted to pH 3.5 with formic acid. This buffer was then diluted 10-fold with either water or acetonitrile to make aqueous and organic mobile phases, respectively. For doxylamine and bretylium tosylate, the mobile phase was 0.1% trifluoroacetic acid (TFA) in water or acetonitrile. Typical gradients started at 10% organic and changed linearly over 3 min to 100% organic at a flow rate of 300 μL/min. Total run times were 4.5 min. Rhodamine 123 was detected by using fluorescence microplate reader (BMG Laboratory) with 492 and 538 nm for excitation and emission, respectively.

2.5. Statistic analysis

Bi-directional cell permeability experiments were performed in quadruplicate; rat brain perfusion study was performed in four–eleven samples, and the data were presented as the mean ± S.E. Student's *t*-test was performed to determine statistical significance between groups in the absence and presence of CsA ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, respectively).

3. Results

The compounds used in this study were divided into two categories, i.e., CNS-positive and CNS-negative. CNS-positive compounds include drugs whose site of pharmacological effects is located in the CNS and compounds known to cross the BBB (Table 1). CNS-negative compounds are those whose pharmacological effects are not in the CNS, or are known not to cross the BBB. The first observation was that CNS-positive compounds in Table 2 exhibited much higher Papp (A–B) values ranging from 3.4×10^{-6} to 20.2×10^{-6} cm/s, whereas CNS-negative compounds in Table 3 showed Papp (A–B) values ranging from 0.028×10^{-6} to 0.83×10^{-6} cm/s. Statistically, there was a significant difference between the lowest Papp (A–B) value of CNS-positive compounds and the highest Papp (A–B) of CNS-negative group ($p < 0.001$). These results suggest that the MDR-MDCK cell line is able to distinguish the two classes of compounds in terms of their CNS penetration. In the work reported by Mahar Doan et al. (2002) antipyrine and doxylamine were categorized in non-CNS-indicated class. Similarly, caffeine was classified into CNS-negative group based on the measured permeability in the PAMPA-BBB system (Di et al., 2003). In the present study, antipyrine, doxylamine and caffeine showed high Papp (A–B) values in MDR-MDCK cells suggesting high brain uptake. To resolve this apparent discrepancy the brain uptake rates of these compounds were determined in the rat brain perfusion system (Table 4). Results from the brain perfusion study further support that the MDR-MDCK cell line can be used as an in vitro tool for evaluation of brain uptake potential of NCEs (new chemical entities).

The second observation was that some compounds in both Tables 2 and 3 displayed active efflux (Papp (B–A)/Papp (A–B) greater than 3), indicating pos-

Table 4
Brain uptake rates of selected compounds in rat brain perfusion model

Compounds	Brain uptake rate (pmol/g/s/μM)	
	Without CsA	With CsA
CNS-positive		
Antipyrine	4.54 ± 0.90	N.D.
Caffeine	18.3 ± 7.2	N.D.
Desipramine	16.92 ± 3.44	N.D.
Doxylamine	2.19 ± 0.73	N.D.
CNS-negative		
Digoxin	0.17 ± 0.05	0.32 ± 0.14*
Lincomycin	0.13 ± 0.10	N.D.
Quinidine	0.24 ± 0.06	4.70 ± 1.63***

N.D.: Not determined; rat brain perfusion data were expressed as mean ± S.D., $n = 4$–11 for each determination. Student's *t*-tests were performed between the brain uptake rates in the absence and presence of CsA.

* $p < 0.05$.

*** $p < 0.001$.

sible involvement of efflux mechanisms, e.g. P-gp. Generally, most compounds that exhibited efflux were found in the CNS-negative group. However, there were two exceptions to this observation: desipramine and doxylamine. These amine drugs showed high Papp (B–A)/Papp (A–B) ratio despite having a high Papp (A–B). The data implies that although some compounds are susceptible to efflux mechanisms they might still be able to penetrate BBB if their Papp (A–B) values are higher than certain threshold value. Under the experimental conditions in the present study, this threshold value appears to be 3×10^{-6} cm/s.

Among the compounds that showed low Papp (A–B) values (Table 3), eight compounds: digoxin, dipyridamole, etoposide, labetalol, loperamide, quinidine, rhodamine 123, and vinblastine, demonstrated high efflux ratios (≥9) and high Papp (B–A) values ($\geq 1.80 \times 10^{-6}$ cm/s), indicating that their CNS penetration was greatly hampered by P-gp and possible other efflux proteins. One strategy to enhance the transport of drugs that are substrates for efflux proteins is to inhibit efflux proteins. To assess the feasibility of this approach, the permeability of these eight compounds was further evaluated by using MDR-MDCK cells in the absence and presence of the P-gp inhibitor, cyclosporin A (CsA). The results of the bi-directional penetration of compounds in the absence or presence of CsA are presented in Fig. 1.

In the absence of CsA, these eight compounds were categorized into three different groups based on their measured Papp (B–A) values. **Panel A** included dipyridamole, loperamide and quinidine with high Papp (B–A) values. **Panel B** contained digoxin, labetalol and vinblastine with moderate Papp (B–A) values. **Panel C** enclosed etoposide and rhodamine 123 with low Papp (B–A) values. CsA greatly reduced the efflux ratio (to nearly 1) and Papp (B–A) values of all tested compounds ($p < 0.001$). Reduction of Papp (B–A) suggested that P-gp plays a key role in the transmembrane movement of these compounds. Interestingly, the presence of CsA significantly increased Papp (A–B) in all **Panel A** compounds ($p < 0.001$): dipyridamole (from 0.30×10^{-6} to 20.09×10^{-6} cm/s), loperamide (from 0.18×10^{-6} to 12.52×10^{-6} cm/s) and quinidine (from 0.34×10^{-6} to 10.77×10^{-6} cm/s). On the other hand, CsA had a weaker effect on the Papp (A–B) of compounds in **Panels B and C**: moderate increment in etoposide (from 0.13×10^{-6} to 0.33×10^{-6} cm/s, $p < 0.01$), labetalol (from 0.30×10^{-6} to 1.34×10^{-6} cm/s, $p < 0.01$) and vinblastine (from 0.40×10^{-6} to 1.23×10^{-6} cm/s, $p < 0.01$), and even weaker enhancement in digoxin ($p < 0.05$) and no obvious effect on rhodamine 123 ($p > 0.05$). The effects of CsA on the permeability of these P-gp substrates observed in the present study were similar to the results reported by other groups using cell models other than MDR-MDCK. Thakker presented that the Papp (A–B) of rhodamine 123 and digoxin measured in Caco-2 cells did not increase after the cells were treated with GW918, a potent P-gp inhibitor (Thakker, 2004). Mizuno et al. (2003) classified the P-gp substrates into three categories based on the effects of P-gp inhibitors on their measured Papp in Caco-2 cells. Under this classification, both rhodamine-123 and digoxin were classified into Class C, in which the increment in Papp (A–B) is insignificant in the presence of a P-gp inhibitor (Mizuno et al., 2003).

These results suggest that P-gp inhibition by CsA could enhance brain uptake for compounds with high Papp (B–A) values, such as quinidine, but had little effects on a compound with moderate Papp (B–A), digoxin. To confirm the in vitro findings, rat brain perfusion was performed on quinidine and digoxin with and without CsA, as shown in Table 4. Co-perfusion with CsA greatly enhanced brain uptake rate for quinidine (from 0.24 to 4.70 pmol/g/s/μM, $p < 0.001$). On

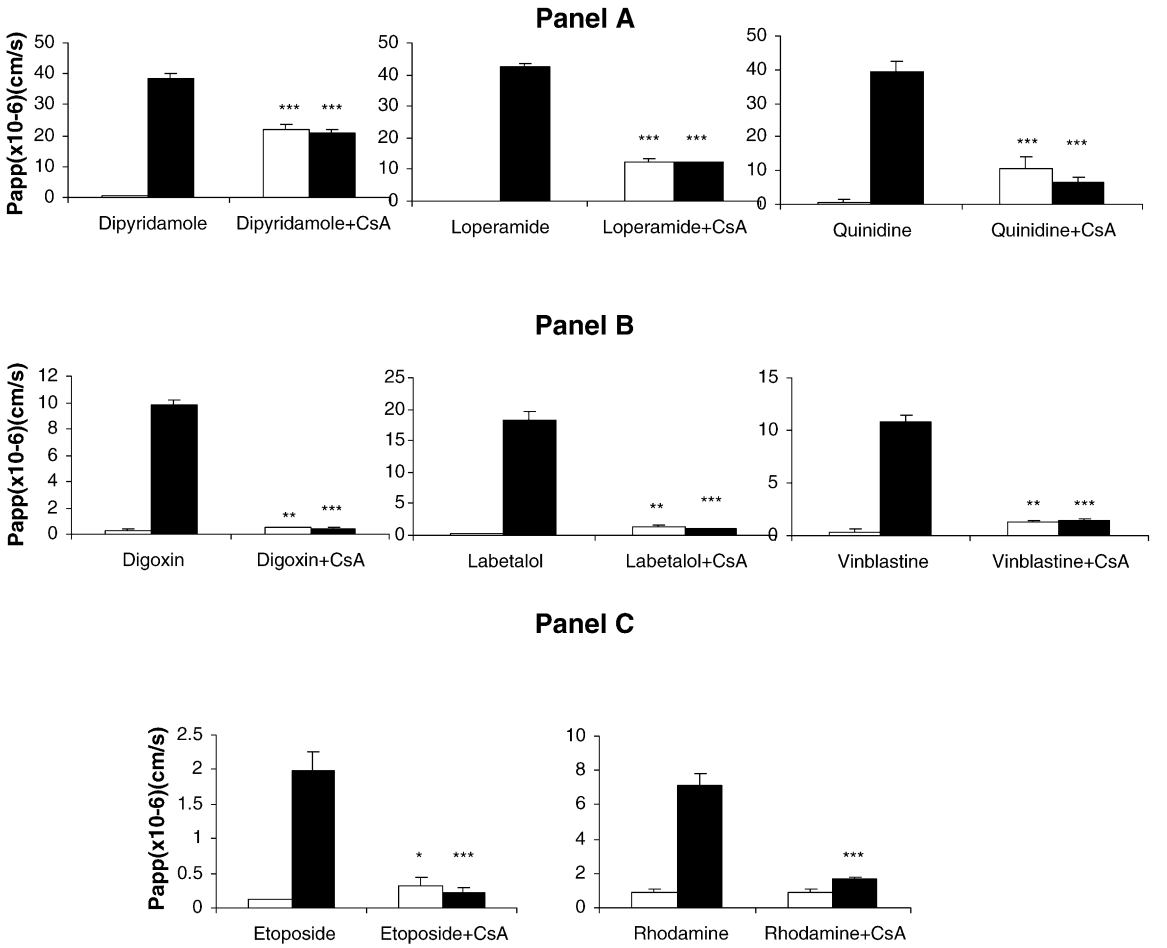

Fig. 1. Permeability coefficients of drugs across MDR-MDCK cells in the absence and presence of CsA. Based on the absolute Papp (B–A) values in the absence of CsA, eight compounds were divided into three panels. **Panel A**: dipyridamole, loperamide and quinidine, with Papp (B–A) approximately 40×10^{-6} cm/s. **Panel B**: digoxin, labetalol and vinblastine, with Papp (B–A) between 10 and 20×10^{-6} cm/s. **Panel C**: etoposide and rhodamine 123, with Papp (B–A) lower than 10×10^{-6} cm/s. The values were designated as mean ± standard deviation ($n = 4$) and Student's *t*-tests were performed between the Papp values (same direction) in the absence and presence of CsA. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Open bar: A–B direction; filled bar: B–A direction.

the other hand, the presence of CsA resulted only in a slight increment in digoxin brain uptake (from 0.17 to 0.32 pmol/g/s/μM, $p < 0.05$). Therefore, in vivo brain uptake results are in good agreement with in vitro MDR-MDCK results.

4. Discussion

Finding a suitable in vitro model to study brain drug delivery has remained one of the most formidable challenges in the pharmaceutical industry. It is not easy to simulate in vivo physiological characteristics of the BBB, such as high expression of P-gp and other efflux proteins, which might be responsible for low drug permeation into the brain. MDCK cells transfected with the P-gp gene exhibited high transepithelial electrical resistance (in the range of 1800–2200 $\Omega\,cm^2$). There has been no information about whether MDR-MDCK formed a monolayer after 8 days culture or the presence of junction proteins in these cells. However, based on the results presented by other investigators, MDCK-

I (strain I with high TEER) formed a monolayer after 7 days seeding on filter membrane (Weinstein et al., 2004). Claudin-1, -4 and occludin were found in MDCK-I cells (Sjo et al., 2003); ZO-1 was also detected in MDCK-I cells (Wu et al., 2000). Since transfecting MDCK-I cells with a retrovirus carrying human multi-drug resistance cDNA that encodes P-gp generated MDR-MDCK cells, it is reasonable to expect that MDR-MDCK also form a monolayer after 8 days growth, and express these tight junction proteins. The uniqueness of the MDR-MDCK cells (i.e., P-gp expression and high transepithelial electrical resistance) makes them a potentially attractive model for BBB permeability screen of NCEs. The purpose of the present study was to test the hypothesis that the MDR-MDCK cells could be used as an in vitro blood–brain barrier tool for screening potential CNS drugs.

The study was carried out in two-steps. First, 28 compounds (27 out of 28 are marketed drugs) were tested using the MDR-MDCK cells in terms of bi-directional transport across cell monolayers. The drugs that intentionally target CNS (CNS-positive), such as amitriptyline, caffeine, carbamazepine, chlorpromazine, doxylamine, fluoxetine (Prozac®) and guanfacine showed values of Papp (A–B) higher than 3×10^{-6} cm/s (i.e., ranging from 3.39 to 20.67×10^{-6} cm/s). The measured Papp (A–B) value of indomethacin, a drug known to have side effects associated with CNS uptake, was 13.67×10^{-6} cm/s. This value is comparable to that of compounds in the CNS-positive group. On the other hand, the Papp (A–B) of drugs that target peripheral sites and have no indication of CNS uptake exhibited low values (i.e., ranging from 0.028 to 0.83×10^{-6} cm/s). These results show a good correlation between the observed MDR-MDCK permeability coefficients of compounds and their in vivo CNS effects. These observations may reflect the ability of MDR-MDCK cells to correctly identify the CNS penetration potential of drug candidates during the drug discovery process. Therefore, the MDR-MDCK cell model may serve as a convenient tool to help guide drug discovery in terms of CNS penetration of NCEs. This process should not only help estimate the brain penetration potential of compounds whose brain uptake is required for therapeutic efficacy, but also may permit to raise awareness about the potential liability of compounds whose brain uptake is undesirable and may lead to CNS toxicity or CNS-associated side effects. Under our experimental conditions, if the measured permeability coefficient of a compound is greater than 3×10^{-6} cm/s, it is highly likely that this compound would be efficient at crossing the BBB and reaching the CNS.

Compared with the results presented by Mahar Doan et al. (2002), two things are worth noting. First, the threshold value for high BBB permeation obtained in the present study is much lower than 15×10^{-6} cm/s, proposed by Mahar Doan et al. (2002). This discrepancy may result from different cell culture conditions. For instance, MDR-MDCK cells used in their study were seeded at 300,000 cells/cm^2 and were ready for use after 3 days, while the seeding density of MDR-MDCK in our study was 60,000 cells/cm^2 and the study initiated 7 days post-seeding.

Secondly, Mahar Doan et al. (2002) suggested that a potential CNS drug candidate should have efflux ratio less than 2.5. In our study, we think that efflux ratio is an important factor to be considered in the CNS drug development, but not the determinant factor in all cases. For instance, both desipramine and doxylamine showed high Papp (B–A)/Papp (A–B) ratio (6.7 and 11, respectively), but their Papp (A–B) (5.56×10^{-6} and 3.39×10^{-6} cm/s) exceeded the threshold value for high BBB penetration, 3×10^{-6} cm/s. Therefore, they were classified as CNS-positive compounds. The rat brain perfusion experiments with desipramine and doxylamine also supported that these two compounds belong to the CNS-positive group (Table 4).

It should be mentioned that a Papp (A–B) in the MDR-MDCK cell lines of NCEs intended for a CNS indication lower than 1×10^{-6} cm/s does not necessarily disqualify a drug candidate as a potential CNS-acting drug. Other parameters, including Papp (B–A) and efflux ratios, should be taken into consideration to judge the potential CNS uptake of the NCEs. We did not find any permeability value in the range of $1–3 \times 10^{-6}$ cm/s. This is probably due to the limited number of compounds studied. Although MDR-MDCK cells can be used as a valuable tool to quickly evaluate CNS uptake potential, it is not the only technique available. Based on the present information, we think that the brain uptake potential of compounds with MDR-MDCK Papp in the $1–3 \times 10^{-6}$ cm/s range should be evaluated using a second method. For this purpose, in vivo or in situ models are the most reliable

way to determine the brain uptake potential of this type of compounds.

In Table 3, several drugs exhibited large Papp (B–A) values ($>1.8 \times 10^{-6}$ cm/s) and high efflux ratios (>10). Both high Papp (B–A) and large efflux ratio suggested that P-gp or other efflux proteins restricted permeation of drugs. Thus, in the second part of study, the permeability of eight compounds known to be P-gp substrates were further determined in MDR-MDCK cells in the presence of the known P-gp inhibitor, CsA. The presence of CsA reduced the efflux ratio to about 1 for all eight compounds.

CsA and its derivatives also have been used to reduce drug-resistance in cancer cells and to enhance the permeation of drugs (Twentyman, 1992; del Moral et al., 1998). In the present study, inhibition of P-gp with CsA greatly enhanced the absorptive (A–B) flux of compounds, such as dipyridamole, loperamide and quinidine. This effect indicates that membrane penetration by these compounds can be enhanced in vivo by P-gp-inhibition. In contrast, P-gp inhibition decreased the secretory (B–A) permeation of digoxin, etoposide, labetalol and rhodamine 123, but showed little enhancement of their associated absorptive permeation. These in vitro findings suggest that inhibition of B–A permeation by a P-gp inhibitor does not necessarily lead to an increase in BBB penetration. This suggestion is supported by results of in vivo brain uptake studies. Thus, P-gp may not be the crucial factor in determining the BBB permeability of these compounds. Instead, intrinsic membrane permeation of these compounds might be the main barrier to CNS penetration. In addition, other efflux proteins might play a role in their low absorptive penetration. P-gp inhibition would not enhance brain penetration by compounds with low intrinsic membrane permeability, or compounds whose BBB efflux is mediated by other efflux proteins (e.g. MRP2, BCRP, etc.).

5. Conclusion

Among the drugs tested in the study, the CNS-positive drugs showed high absorptive transport across the MDR-MDCK cells, whereas CNS-negative drugs showed low absorptive transport. The distinction between these two classes of drugs is obvious and there is no overlap: the passive Papp (A–B) of CNS-positive compounds is greater than 3×10^{-6} cm/s and Papp (A–B) of CNS-negative compounds is less than 1×10^{-6} cm/s. The data suggest that the MDR-MDCK cell line could be used as a quick BBB model to aid drug discovery. The study of P-gp inhibition also shed light on transport mechanisms and factors that regulate transmembrane drug movements. These results suggest that P-gp might be the dominant factor on the permeation of some of the P-gp substrate drugs used in this study. For other drugs, however, factors such as intrinsic membrane permeability may be more important in limiting CNS penetration. This distinction is very important because, in many cases, interaction with P-gp might be considered the sole or main reason that a compound does not reach the brain, when in reality, P-gp may play an insignificant role in CNS penetration.

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