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Research Paper

A Fluorometric Screening Assay for Drug Efflux Transporter Activity in the Blood-Brain Barrier

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Purpose. To examine the capability of a fluorometric assay to identify and characterize the drug efflux interactions of a broad spectrum of drug agents in an *in vitro* model of the blood-brain barrier (BBB).
Methods. Various concentrations of drug agent (1, 10, and 100 μM) were evaluated for their effect on the cellular accumulation of the P-glycoprotein (P-gp) probe R123 (3.2 μM), and the mixed P-gp and multidrug resistance-associated protein (MRP) probe, BCECF (1 μM), in bovine brain microvessel endothelial cell (BBMEC) monolayers. Drugs demonstrating a significant effect were further quantitated using an expanded concentration range and a nonlinear regression curve fit to determine the potency (IC_{50}) and efficacy (Imax) of the drug for P-gp and/or MRP.
Results. Several of the 36 therapeutic agents examined showed drug efflux transporter interactions in BBMEC monlayers. Melphalan and risperidone significantly enhanced the accumulation of R123 over control (1.47- and 1.82-fold, respectively) with resulting IC_{50}s of 1.4 and 14.6 μM, respectively. Chlorambucil and valproic acid significantly enhanced the accumulation of BCECF compared to control monolayers (2.02- and 4.01-fold, respectively) with resulting IC_{50}s of 146.1 and 768.5 μM, respectively.
Conclusions. The current study demonstrates the feasibility of a fluorometric assay consisting of R123 and BCECF in assessing the drug efflux interactions of a variety of drugs in the BBB.

KEY WORDS: BCECF; blood-brain barrier; MRP; P-gp.

INTRODUCTION

P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and multidrug resistance-associated protein (MRP) are members of the ATP-binding cassette superfamily of proteins that were first discovered in multidrug-resistant tumor cells (1–4). These proteins actively extrude a broad range of chemically unrelated compounds out of the cell, resulting in decreased drug accumulation and drug response (5). These same transport proteins have been identified in normal tissues including 1) brain endothelial cells (6–11), 2) intestinal epithelial cells (12–16), 3) liver hepatocytes (1,13,16–18), and 4) kidney epithelial cells (4,15,19,20). More recently, various organic anion transporting polypeptides (OATP) and organic anion transporters (OAT) have been identified in a variety of tissues, including the brain, and are thought to play a role in the detoxification of compounds from the body (21). Given their wide range of substrates, expression of P-gp, BCRP, MRP, and OAT(P) in these tissues could dramatically alter the absorption, distribution, and elimination of variety of drugs.

The expression of P-gp, BCRP, MRP, and OAT(P) in the capillary endothelium of the brain could contribute significantly to the restrictive nature of blood-brain barrier (BBB). The BBB reduces the permeability of many compounds to the brain, protecting sensitive tissue from potentially toxic drug concentrations. However, the BBB also prevents many compounds from achieving therapeutic levels in the brain, subsequently minimizing drug efficacy. Thus, the biological responses to drugs in the brain are dependant on BBB penetration. Identification of compounds whose brain permeability may be affected by P-gp, BCRP, MRP, and OAT(P) early in the drug development process would provide valuable information concerning the central nervous system (CNS) efficacy and/or toxicity of a drug.

Previously, we characterized the drug efflux interactions of 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) and its fluorescent free acid (BCECF) (22). The nonfluorescent lipophilic ester, BCECF-AM, interacts with P-gp and BCRP, while its fluorescent anionic metabolite, BCECF, is a substrate for MRP and potentially other organic anion transporters (22). The multisubstrate nature of BCECF-AM and BCECF is a property that can be exploited in order to evaluate the drug efflux activity of tissues expressing multiple types of drug efflux transporters, such as the BBB. The purpose of the current study was to examine the feasibility of a fluorometric assay consisting of rhodamine 123 (R123) and BCECF-AM in assessing the drug efflux interactions of a broad spectrum of drugs.

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MATERIALS AND METHODS

Materials and Reagents

2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) and its free acid (BCECF) were purchased from Molecular Probes (Eugene, OR, USA). Rhodamine 123 (R123), indomethacin, azidothymidine (AZT), 2',3'-dideoxyinosine (DDI), 2',3'-dideoxycytidine (DDC), 2',3'-didehydro-3'-deoxythymidine (D4T), paclitaxel (taxol), methotrexate, cisplatin, etoposide (VP-16-213), 5-fluorouracil (5-FU), carmustine (BCNU), melphalan, chlorambucil, carboplatin, fluoxetine hydrochloride, valproic acid sodium salt, phenytoin, nortriptriptyline hydrochloride, risperidone, gabapentin, zimelidine dihydrochloride, pergolide methanesulfonate, tacrine hydrochloride, cocaine hydrochloride, phencyclidine hydrochloride, morphine sulfate pentahydrate, morphine-6-β-D-glucuronide dihydrate (M6G), morphine-3-β-D-glucuronide (M3G), D-amphetamine sulfate, ketamine hydrochloride, nicotine, bovine fibronectin, and equine serum were purchased from Sigma Chemical Company (St. Louis, MO, USA). Gemcitabine was provided by Eli Lilly and Company (Indianapolis, IN, USA). An injectable formulation of doxorubicin was purchased from the UNMC hospital (Omaha, NE, USA). GF120918 was provided by GlaxoSmithKline (Research Triangle Park, NC, USA). Cyclosporin A was purchased from Alexis Corporation (San Diego, CA, USA). Tissue culture plates, minimum essential media (MEM), Ham's F-12, Triton-X 100, and rat tail collagen was purchased from Fisher (St. Louis, MO, USA). Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Indianapolis, IN, USA).

Cell Isolation and Culturing

Primary bovine brain microvessel endothelial cells (BBMECs) were isolated from the gray matter of fresh bovine cerebral cortices using enzymatic digestion and centrifugal separation methods as previously described (23). The BBMECs were seeded (50,000 cells/cm^2) on collagen-coated, fibronectin-treated, 24-well polystryene tissue culture plates. The culture media consisted of: 45% minimum essential medium, 45% Ham's F-12 nutrient mix, 10 mM HEPES, 13 mM sodium bicarbonate, 50 μg/ml gentamicin, 10% equine serum, 2.5 μg/ml amphotericin B, and 100 μg/ml heparin. The BBMECs were cultured in a humidified 37°C, 5% CO_2 incubator, with media replacement occurring every other day until the monolayers reached confluency (approximately 10–14 days).

Drug Screening Assay

For the initial drug screening studies, confluent BBMEC monolayers were pre-treated in pH 7.4 Tyrodes balanced salt solution (TBSS): 136 mM NaCl, 2.6 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 5.56 mM D-Glucose, and 5 mM HEPES; in the presence or absence of various therapeutic agents (1–100 μM) or cyclosporin A (3.2 μM) and indomethacin (10 μM) for 30 min at 37°C. Due to a lack of solubility, paclitaxel and methotrexate could only be examined up to 10 μM. The P-gp inhibitor, cyclosporin A, and the nonspecific MRP-related transport inhibitor, indomethacin, were used in combination as a positive control for the BCECF studies, while cyclosporin A alone was used as a positive control for the R123 studies. Following the pretreatment period, solutions were removed and the monolayers were incubated in TBSS with BCECF-AM (1 μM) or R123 (3.2 μM) in the presence or absence of various concentrations of drug agents or cyclosporin A and indomethacin for 60 min at 37°C. After the incubation period, solutions were removed and the monolayers were washed three times with ice-cold phosphate buffered saline (PBS). The cell monolayers were subsequently solubilized in 1% Triton-X 100. Aliquots (100 μl) of the solubilized cell solutions were removed for determination of intracellular BCECF (λex = 505 nm and λem = 535 nm), or R123 (λex = 505 nm and λem = 535 nm) accumulation using a fluorescence spectrofluorophotometer (Shimadzu RF5000; Shimadzu, Kyoto, Japan). The protein content in the samples was determined using the Pierce BCA method, and the data were expressed as the amount of fluorescent probe (nmol) per mg cell protein.

Potency and Efficacy Determinations

In order to determine the potency and efficacy of each drug agent for P-gp or MRP-related transport, confluent BBMEC monolayers were pre-treated in TBSS (pH 7.4) in the presence or absence of various concentrations of drug agents and/or GF120918 (3.2 μM) for 30 min at 37°C. Following the pretreatment period, solutions were removed and the monolayers were incubated in TBSS with BCECF-AM (1 μM) in the presence or absence of various concentrations of drug agents and GF120918 for 60 min at 37°C to determine MRP-related transport activity. Separate studies were performed using BBMEC monolayers incubated in TBSS with R123 (3.2 μM) in the presence or absence of various concentrations of drug agents for 60 min at 37°C to determine P-gp activity. After the incubation period, solutions were removed and the monolayers were washed three times in ice-cold phosphate buffered saline (PBS). The cell monolayers were subsequently solubilized in 1% Triton-X 100. Aliquots (100 μl) of the solubilized cell solutions were removed for determination of intracellular BCECF or R123 accumulation and protein content using the analytical conditions specified above. IC_{50} values and efficacies were determined using a sigmoidal dose-response nonlinear regression curve fit of the experimental data performed by GraphPad Prism version 3.02 (GraphPad Software, San Diego, CA, USA). In the context of this paper, the potency of a drug for P-gp or MRP-related transport is the concentration of drug required to half-maximally enhance the cellular accumulation of R123 and/or BCECF above that observed in the absence of drug, and is indicated by the IC_{50} value. The efficacy of a drug for P-gp or MRP-related transport is the magnitude by which the drug is able to maximally enhance the cellular accumulation of R123 and/or BCECF above that of monolayers exposed to the fluorescent probe alone, and is represented by Imax.

Statistical Analyses

Statistical evaluations were performed using a one-way ANOVA coupled with Fisher's post-hoc comparisons.

RESULTS

Drug Screening Assay

A total of 36 agents were examined in the initial drug screen. These agents were classified as: 1) antiviral, 2) anti-

cancer, 3) CNS-acting, and 4) drugs subject to abuse. The maximal response of each agent along with the responses of known inhibitors of P-gp (cyclosporin A) and MRP-related transport (indomethacin) on BCECF and R123 accumulation in BBMEC monolayers is shown in Figs. 1-4. Cyclosporin A alone or in combination with indomethacin significantly increased the cellular accumulation of R123 (2-fold) and BCECF (5.5-fold) over that of control monolayers receiving only fluorescent probe. Of the antiviral agents examined, roscovitine was the only compound demonstrating significant activity, increasing both R123 and BCECF above the control groups (Fig. 1). Ganciclovir and several of the nucleoside compounds showed moderate effects on BCECF accumulation, but these responses did not reach statistical significance.

For the anticancer agents (Fig. 2), doxorubicin and taxol greatly affected the accumulation of both R123 and BCECF. Taxol elevated R123 intracellular concentrations (2-fold) equivalent to those observed in the presence of the positive control, cyclosporin A. While not as dramatic, doxorubicin also increased R123 accumulation in the BBMEC (1.4-fold) over control. Doxorubicin and taxol were similar in their effect on BCECF accumulation (2.1 and 2.8-fold, respectively over control). The only other anticancer agent showing significant enhancement of R123 accumulation was melphalan. Interestingly, chlorambucil, a structurally similar agent to melphalan, was inactive in the R123 assay, but highly active in the BCECF assay. Other anticancer agents significantly increasing BCECF accumulation include methotrexate (2.5-fold enhancement) and cisplatin (2-fold enhancement) (Fig. 2). The selective effects of these compounds on BCECF accumulation suggests a primary interaction with MRP-related transport, and not P-gp.

The accumulation of R123 and BCECF in the presence of various CNS-acting agents is represented in Fig. 3. The antipsychotic risperidone significantly elevated both R123 and BCECF accumulation (1.8 and 2.3-fold, respectively) compared to control, suggesting an interaction with P-gp and potentially MRP-related transport. Treatment with the anticonvulsant valproic acid resulted in the highest accumulation of BCECF of any of the 36 drugs examined (4 –fold). The cognitive agent tacrine also significantly increased the accumulation of BCECF compared to control (2.4-fold). These results, along with the observation that neither valproic acid nor tacrine affected R123 accumulation, suggest these compounds have little interaction with P-gp, but may have signifi-

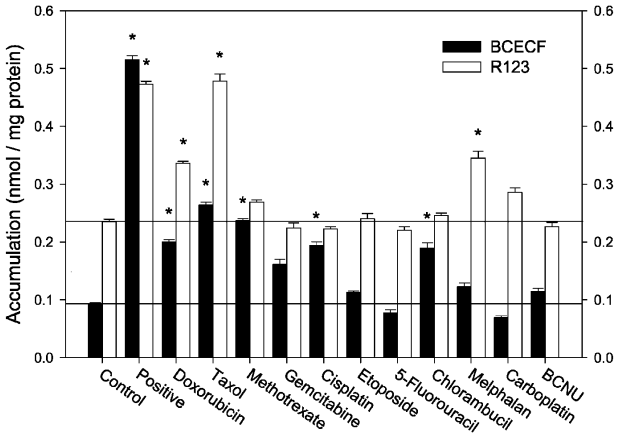

Fig. 2. Intracellular accumulation of BCECF or R123 in the absence or presence of various anticancer agents at 60 min in BBMEC confluent monolayers. The accumulation in the presence of 100 μM drug is presented with the exception of taxol and methotrexate (10 μM). Control represents BCECF or R123 accumulation in the absence of drug. Positive represents the combination of cyclosporin A (3.2 μM) and indomethacin (10 μM) for BCECF and cyclosporin A alone for R123. Values represent the mean ± SEM (n = 9). *$p < 0.05$ compared to control monolayers at the same incubation time as determined by a one-way ANOVA and Fisher's *post hoc* comparisons. BCNU, carmustine.

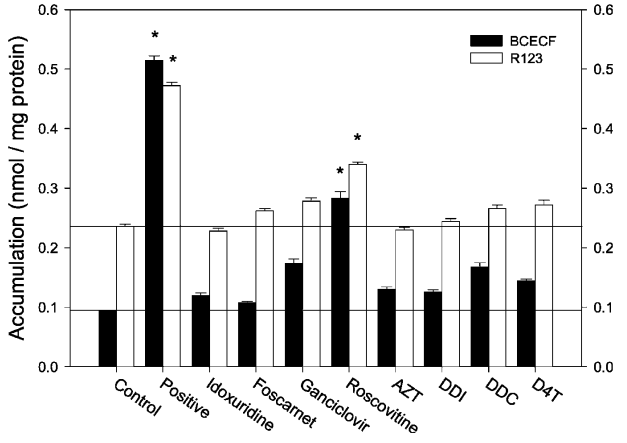

Fig. 1. Intracellular accumulation of BCECF or R123 in the absence or presence of various antiviral agents (100 μM) at 60 min in BBMEC confluent monolayers. Control represents BCECF or R123 accumulation in the absence of drug. Positive represents the combination of cyclosporin A (3.2 μM) and indomethacin (10 μM) for BCECF and cyclosporin A alone for R123. Values represent the mean ± SEM (n = 9). *$p < 0.05$ compared to control monolayers at the same incubation time as determined by a one-way ANOVA and Fisher's *post hoc* comparisons. AZT, azidothymidine; DDI, 2',3'-dideoxyinosine; DDC, 2',3'-dideoxycytidine; D4T, 2',3'-didehydro-3'-deoxythymidine.

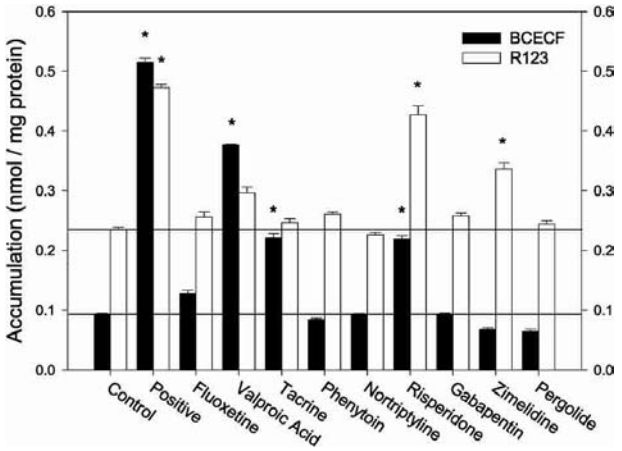

Fig. 3. Intracellular accumulation of BCECF or R123 in the absence or presence of various central nervous system-acting agents (100 μM) at 60 min in BBMEC confluent monolayers. Control represents BCECF or R123 accumulation in the absence of drug. Positive represents the combination of cyclosporin A (3.2 μM) and indomethacin (10 μM) for BCECF and cyclosporin A alone for R123. Values represent the mean ± SEM (n = 9). *$p < 0.05$ compared to control monolayers at the same incubation time as determined by a one-way ANOVA and Fisher's *post hoc* comparisons.

cant interactions with the MRP-related transporters. The antidepressant zimelidine was capable of significantly enhancing the accumulation of R123 (1.4-fold), but this apparent P-gp interaction was not enough to affect the accumulation of BCECF. The antiepileptic phenytoin, the antidepressant nortriptyline, and the antiparkinsonian agent pergolide, did not alter the accumulation of R123 or BCECF from that of control.

In the drugs subject to abuse class (Fig. 4), many of the drugs showed minor increases in BCECF accumulation, though none of the observations were statistically significant. With respect to R123, phencyclidine (PCP), the morphine analog morphine-3-β-D-glucuronide (M3G), and nicotine all significantly enhanced R123 accumulation compared to control. Phencyclidine increased intracellular R123 levels (1.8-fold) near those observed in the presence of the positive control, cyclosporin A (2-fold). The elevations of intracellular R123 in the presence of M3G and nicotine were more modest at 1.5- and 1.4-fold over control, respectively. The influence of these compounds on R123 accumulation but not BCECF accumulation suggests they interact with P-gp, but have minimal influence on MRP-related transport.

Potency and Efficacy Determinations

Due to the multi-substrate nature of the BCECF probe (P-gp, BCRP, and MRP-related transport), it is not possible to determine the potency and/or efficacy of a drug for MRP-related transport from the initial BCECF drug screen. However, if P-gp and BCRP activity in the BBMEC could be completely inhibited, then BCECF could be used as a probe for MRP-related transport activity. In order to accomplish this, the concentration of the P-gp (24) and BCRP (25) inhibitor, GF120918, necessary to completely ablate P-gp and BCRP function (IC_{100}) in the BBMEC was determined. Accumulation of the P-gp and BCRP substrate, R123, was examined in the presence of various concentrations of GF120918 in BBMEC monolayers at 60 min (Fig. 5). A plateau in R123 accumulation was observed in the presence of 3.2 μM GF120918 indicating maximal P-gp inhibition (IC_{100}). Further confirmation was evident when drugs known to inhibit P-gp or BCRP, administered in conjunction with 3.2 μM GF120918 in the BBMEC, produced no further change in R123 or BCECF accumulation compared to that in the GF120918 treatment group (data not shown). Based on these studies and the maximal P-gp inhibition ranges of GF120918 reported in the literature for other P-gp substrates using various P-gp-expressing cells (approxiamately 1 μM) (24,26,27), 3.2 uM GF120918 appeared to be a satisfactory concentration necessary to maximally inhibit P-gp/BCRP function in the BBMEC.

Compounds that displayed the largest magnitude of drug efflux activity in either the R123 or BCECF assays were selected for further evaluation in order to quantitate and characterize their impact on drug efflux activity in the BBMEC. The cellular accumulation of R123 and BCECF in the presence of 3.2 μM GF120918 was examined over an expanded concentration range for each compound in order to determine the average potency and efficacy of the various drug agents for either P-gp or MRP-related transport in the BBMEC. The theoretical profile of BCECF accumulation in the presence of compounds with varying drug efflux interactions is shown in Fig. 6. The point labeled control represents the theoretical accumulation of BCECF under control conditions at 60 min (absence of inhibitor or drug). Following exposure to GF120918, BCECF accumulation would increase due to the pharmacological inhibition of P-gp and BCRP. A drug that has only P-gp and/or BCRP interactions would not be expected to increase BCECF accumulation above that of 3.2 μM GF120918 treatment alone (drug A in Fig. 6). A drug that interacts with MRP-related transport would be expected to elevate the accumulation of BCECF above that of 3.2 μM GF120918 treatment alone (drug B in Fig. 6).

Using this approach, BCECF and R123 accumulation profiles in the presence of each drug were examined in BBMEC monolayers and the resulting data fitted to a sigmoidal dose-response nonlinear regression curve. Representative concentration response curves for a predominantly MRP interacting agent (chlorambucil) and a predominantly P-gp in-

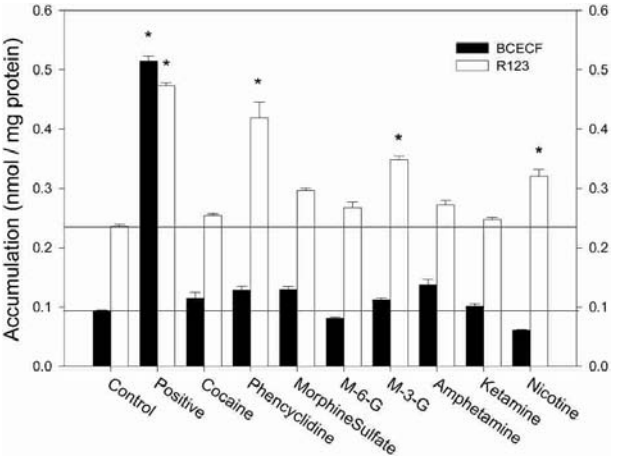

Fig. 4. Intracellular accumulation of BCECF or R123 in the absence or presence of various drugs subject to abuse (100 μM) at 60 min in BBMEC confluent monolayers. Control represents BCECF or R123 accumulation in the absence of drug. Positive represents the combination of cyclosporin A (3.2 μM) and indomethacin (10 μM) for BCECF and cyclosporin A alone for R123. Values represent the mean ± SEM (n = 9). *p < 0.05 compared to control monolayers at the same incubation time as determined by a one-way ANOVA and Fisher's *post hoc* comparisons. M6G, morphine-6-β-D-glucuronide dihydrate; M3G, morphine-3-β-D-glucuronide.

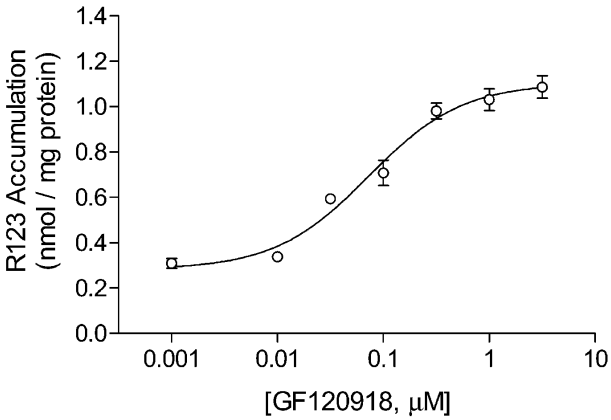

Fig. 5. Intracellular accumulation of R123 in the presence of various concentrations of GF120918 at 60 min in BBMEC confluent monolayers. Values represent the mean ± SEM (n = 3).

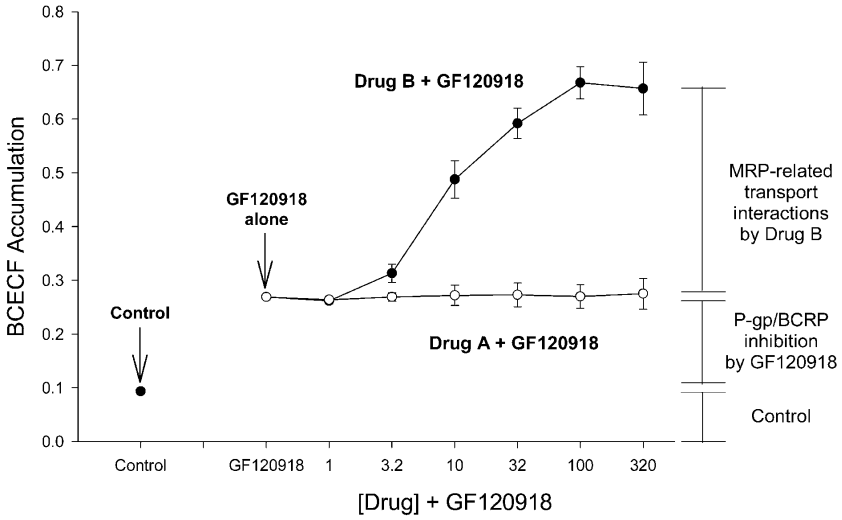

Fig. 6. Theoretical accumulation profiles of BCECF in the presence of GF120918 alone or in combination with drugs of varying drug efflux activity.

teracting agent (risperidone) are shown in Fig. 7. Potency and efficacy data for all compounds identified as having drug efflux activity in the initial screen are provided in Table I. Chlorambucil in the presence of 3.2 μM GF120918 significantly enhanced BCECF accumulation (2.73-fold) over GF120918 treatment alone with maximal inhibition occurring at 1mM chlorambucil (Fig. 7A). Conversely, R123 accumulation was not affected by chlorambucil at any of the concentrations examined (Fig. 7A). Risperidone, on the other hand, did not alter the accumulation of BCECF in the presence of GF120918, but did significantly enhance the accumulation of R123 (1.82-fold) compared to control conditions (Fig. 7B). Of the drugs displaying activity for P-gp, the rank order potencies were GF120918 >> melphalan > risperidone > roscovitine > phencyclidine. The rank order efficacies of these drugs with P-gp were GF120918 >> risperidone > roscovitine > melphalan > phencyclidine. With respect to MRP-related transport, the rank order potencies were indomethacin > cisplatin > chlorambucil > tacrine. The rank order efficacies for MRP-related transport were indomethacin > chlorambucil > tacrine > cisplatin.

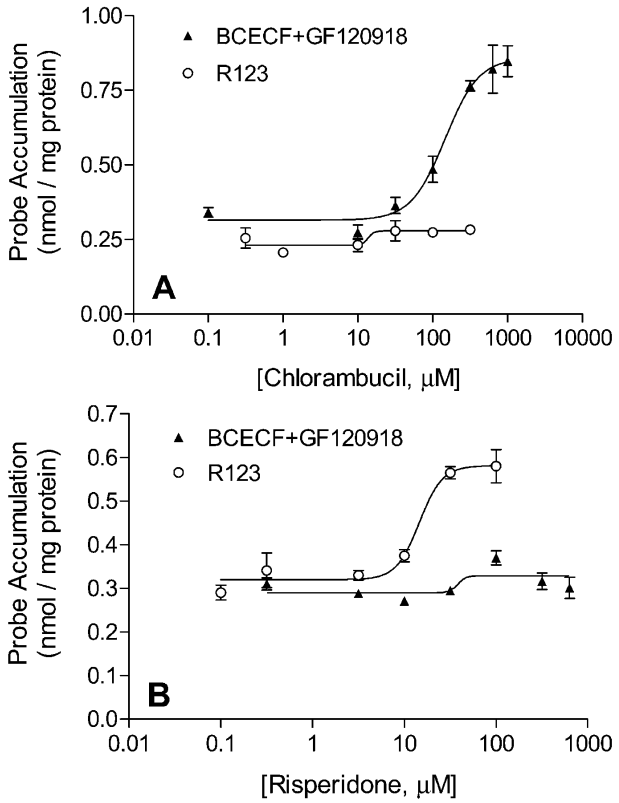

Fig. 7. Intracellular accumulation of R123 or BCECF in combination with GF120918 in the presence of various concentrations of (A) chlorambucil, or (B) risperidone at 60 min in BBMEC confluent monolayers. Values represent the mean ± SEM (n = 3).

DISCUSSION

The current study used the fluorescent probes R123 and BCECF to identify and characterize interactions involving P-gp and MRP-related drug efflux transporters in brain endothelial cells. It should be noted that R123 (28) and the ester form of BCECF (22) are suitable probes for BCRP. Previous studies suggest BCRP does not play a significant role in the accumulation of BCECF (22) or R123 (data not shown) in cultured brain microvessel endothelial cells at time points greater than fifteen minutes. While it may be possible to alter the conditions of the assay to capture BCRP interactions, under the current conditions used (i.e., 60 min), accumulation of R123 and BCECF in the BBMEC primarily reflect P-gp and MRP-related transport activity.

The presence of multiple drug efflux transport systems in the BBB play an important homeostatic and protective role by limiting the entry of many compounds into the brain, as well as accelerating their elimination from the brain. The relatively broad spectrum of compounds recognized by P-gp and MRP presents a challenge in drug development as many therapeutic agents act as either substrates and/or inhibitors of drug efflux transport. Thus, evaluating changes in R123 and BCECF accumulation in the BBMEC represents a simple and rapid method for determining interactions with drug efflux transport systems in the BBB.

The use of R123 as a probe to evaluate P-gp activity is well established (29,30). The initial screening study using

Table I. Potency and Efficacy Determinations for P-gp or MRP-Related Transport in BBMEC

		P-gp			MRP-related transport		
		Potency		Efficacy	Potency		Efficacy
Class	Drug	IC_{50} (μM)	95% CI	Imax[a]	IC_{50} (μM)	95% CI	Imax[a]
Antiviral	Roscovitine	15.07	5.86 to 38.72	16.2	8.53	6.96 to 10.45	17.1
Anticancer	Cisplatin	>1000	NA[c]	5.5	89.07	78.53 to 101.0	21.6
	Chlorambucil	>1000	NA[c]	5.9	146.10	83.83 to 254.5	70.1
	Melphalan[b]	1.40	0.6299 to 3.139	15.7	>1000	NA[c]	10.0
CNS-acting	Tacrine	>1000	NA[c]	7.0	329.0	194.5 to 556.8	53.3
	Risperidone	14.58	4.410 to 48.17	31.8	>1000	NA[c]	4.7
	Valproic acid[b]	>1000	NA[c]	11.4	768.5	265.8 to 1776	215.4
Drugs subject to abuse	Phencyclidine	40.92	29.75 to 56.27	12.3	>1000	NA[c]	6.3
Drug efflux inhibitors	GF120918 (P-gp)	0.072	0.03399 to 0.1543	100		ND[d]	
	Indomethacin (MRP-related)		ND[d]		31.77	11.09 to 91.03	100

[a] Values represent the maximal response of drug expressed as a percentage of the maximal inhibition obtained with GF120918 or indomethacin.
[b] Toxicity apparent at high concentrations.
[c] Not applicable.
[d] Not determined.

R123 in the BBMEC identified several agents as having significant P-gp interactions. These agents included doxorubicin, taxol, and morphine-3-β-D-glucuronide, all well-known P-gp substrates (31–36). Other agents giving positive responses in the R123 assay, such as roscovitine, melphalan, risperidone, zimelidine, phencyclidine, and nicotine, are not readily recognized for their interactions with P-gp. However, it should be noted that a recent study reported P-gp ATPase activity for a number of antipsychotic agents, including risperidone (37). Furthermore, previous studies have suggested P-gp-like transport properties for both melphalan (38,39) and nicotine (40,41). These studies suggest that R123 accumulation in BBMEC monolayers appears to provide a robust and reproducible screen for potential P-gp interactions of drugs in the BBB.

While P-gp is certainly a major determinant of BBB permeability for many therapeutic agents (42,43), other drug efflux transport systems such as MRP, may also contribute to the low brain accumulation of various compounds. Based on previous studies in our laboratory, BCECF accumulation in the BBMEC, unlike R123, was influenced by both P-gp and MRP-related transport (22). Thus, it was hypothesized that analysis of the same series of therapeutic agents in the BCECF drug screen would provide a more complete assessment of drug efflux transporter interactions in the BBB.

In the current study, drugs displaying activity in the BCECF assay could be separated into two distinct groups. There were those agents, first identified in the R123 assay, which increased BCECF accumulation. This group included taxol, doxorubicin, roscovitine, and risperidone. The second group was made up of drugs that increased BCECF accumulation, but showed no activity in the R123 assay. This group included such agents as valproic acid, tacrine, methotrexate, cisplatin, and chlorambucil. The effects of this second group of agents in the BCECF assay, combined with their lack of activity on R123 accumulation, suggest that these agents have predominantly MRP-related transport interactions in the BBMEC. In support of this, it should be noted that MRP-mediated interactions have been reported previously for both valproic acid (44,45) and methotrexate (2,46,47). Furthermore, the observation that cisplatin-resistant cells overexpress MRP and demonstrate reduced levels of cisplatin accumulation compared to drug sensitive parental cell lines is consistent with an MRP-mediated efflux of cisplatin (48). Prior studies have indicated chlorambucil does not interact with MRP1 (49–51); however, metabolites of chlorambucil (i.e., glutathione conjugates) are sensitive to MRP1 transport (49). As a result, the effects observed for chlorambucil in the BCECF assay may be due to an interaction of the metabolites with MRP and not parent drug.

There are several advantages to using R123 and BCECF as quick, qualitative screens for drug efflux activity. First, it allows for the identification of both P-gp and MRP-related transport interactions. Given the evidence in support of MRP expression on the luminal membrane of brain endothelial cells (52,53), such interactions may be important determinants of the brain accumulation of select drugs. In the present study, if only the R123 assay were performed, compounds such as methotrexate and valproic acid, which appear to have predominantly MRP-related transport activity, would have gone undetected. Second, the R123 and BCECF drug screening assays can assist in refining the structure-activity properties of a drug candidate. An example from the current set of compounds is melphalan and chlorambucil, two DNA alkylating agents from the anticancer group. Although structurally similar, melphalan and chlorambucil behave very differently in the R123 and BCECF assays, with melphalan showing P-gp interactions (R123) and chlorambucil displaying MRP-related transport interactions (BCECF). Structure-activity differences were also observed with cisplatin (MRP-related transport) and carboplatin (no interactions detected).

As with any screening assay there are some limitations. A considerable number of agents, identified as having moderate P-gp interactions in the R123 assay, did not give positive results in the BCECF assay (i.e., melphalan, nicotine, and morphine-3-β-D-glucuronide). Thus, if only the BCECF assay were used, there is a chance that drugs having low to moderate interactions with P-gp would be overlooked. While low to

moderate interactions with P-gp may not impact oral drug absorption, such interactions may be sufficient to alter the brain distribution of a compound. This limitation with the BCECF assay can be overcome by using the BCECF assay in conjunction with the R123 assay to provide a more complete assessment of a wide range of P-gp interactions.

An additional consideration in using fluorescent probes such as R123 and BCECF to assess drug efflux transporter activity is the cell permeability of the drugs being screened. Compounds most likely to interact with MRP-related transporters tend to be relatively hydrophilic, and would not enter the cell as easily as the more lipophilic P-gp substrates. Thus, those compounds demonstrating MRP-related transport activity in the present study have appreciable accumulation inside the cell, either through passive diffusion (valproic acid and chlorambucil) or carrier-mediated influx (methotrexate). Similarly, insufficient cellular penetration is a likely explanation for the lack of response observed with agents such as AZT and the morphine analogs in the current drug screening assays. This is not unique to the present study, as others have reported similar limitations in evaluating the drug efflux transport (54) and drug efflux modifying (55) properties of compounds with low permeability.

In addition to providing a quick, qualitative evaluation of potential drug efflux interactions, the present study used the R123 and BCECF accumulation assays to quantitatively assess the P-gp and MRP-related transport interactions of several different agents in the BBMEC. In these studies a larger range of drug concentrations were examined to determine the potency (IC_{50}) and efficacy (Imax) of compounds for P-gp (R123 assay) and MRP-related (BCECF assay) transport. As BCECF is a multi-transporter probe, P-gp activity was maximally inhibited using GF120918. The validity of this approach is demonstrated in Fig. 7 using a compound initially identified as having predominantly MRP-related transport interactions (chlorambucil) and a compound that primarily interacts with P-gp (risperidone).

While the present study is the first to quantitatively determine IC_{50} and Imax values for P-gp and MRP-related transporters in the BBMEC, comparisons to other cells or other drug screening assays is quite favorable. The IC_{50} value of 14.6 μM obtained for risperidone in the R123 assay in the present study is comparable to the Km value of 18 μM reported by Boulton *et al.* in a P-gp ATPase assay (37). Likewise, the IC_{50} values reported in the literature for inhibition of P-gp by GF120918 range from 20 to 100 nM (24,26,27). These values are consistent with the IC_{50} value of 72 nM for GF120918 determined from the R123 assay in the current study. From an MRP standpoint, there is considerably less data with which to compare our current results. Still, indomethacin has been used as a nonspecific inhibitor of MRP-related transport. An IC_{50} of 10-20 μM has previously been reported for indomethacin in MRP1 expressing cells (56). This is comparable to the 31.7 μM IC_{50} value obtained in the current study using BCECF accumulation in BBMEC monolayers, especially considering the BBMEC express more than one isoform of MRP (i.e., MRP1, MRP4, MRP5, MRP6) for which indomethacin will act as an inhibitor (57).

The ability to quantitatively assess drug interactions with P-gp and MRP-related transport in brain endothelial cells adds another dimension to the drug-screening assay presented. By determining IC_{50} values in the R123 and BCECF assays, one can assess the potency of a compound for P-gp and MRP-related transport in the BBB. This provides a method for quantitatively assessing structure-activity relationships and identifying the chemical modifications that minimize drug efflux transport interactions. Furthermore, the IC_{50} values obtained in the *in vitro* drug-screening assay can be compared to known or anticipated therapeutic blood levels of a compound to determine the potential for a clinically relevant interaction. For example, the IC_{50} value obtained in the R123 assay for melphalan (1.4 μM) is below the normal range of plasma values achieved with this drug (based on the 2003 product information for melphalan by GlaxoSmithKline, Research Triangle Park, NC, USA). Thus, one would anticipate that melphalan interactions with P-gp in the BBB may be more of a concern from a therapeutic or toxicity standpoint than that of risperidone, which has an IC_{50} value well above the serum levels normally observed in the clinic (58).

In summary, we report a method for screening potential P-gp and MRP-related transport interactions in the BBB using two different fluorescent probes and primary cultured BBMEC as an *in vitro* model of the BBB. By screening compounds with both R123 and BCECF, a wider range of drug efflux transport interactions can be explored. The assay can be used in a qualitative or quantitative manner to assess P-gp and MRP-related transport activity and may be an important tool for CNS drug discovery groups within the pharmaceutical industry.

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