COMPARISON OF DRUG EFFLUX TRANSPORT KINETICS IN VARIOUS BLOOD-BRAIN BARRIER MODELS

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ABSTRACT:
The present study quantitatively compared the drug efflux transport kinetics of 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) and its fluorescent metabolite 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) in various blood-brain barrier (BBB) models. BCECF-AM was exposed to freshly isolated bovine brain microvessels (BBM), primary cultured bovine brain microvessel endothelial cells (BBMEC), and MDCK-MDR1 cells for 30 min in the presence or absence of the P-glycoprotein (P-gp) inhibitor 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 (GF120918). P-gp transport kinetics were determined indirectly by calculating the difference in BCECF accumulation when P-gp was functional and completely inhibited by GF120918 (3.2 μM). Multidrug resistance-associated protein (MRP) transport kinetics were determined by measuring the amount of BCECF transported out of the cell over time. For P-gp-related transport, K_m values for BCECF-AM were approximately the same in all three models (around 2 μM), whereas the V_max was 4-fold greater in the BBM than in the BBMEC or MDCK-MDR1 cells. For MRP-related transport, K_m values for BCECF varied widely among the three BBB models with a rank order of MDCKII-MDR1 < BBMEC < BBM. Like P-gp, the V_max of BCECF for MRP-related transport was overwhelmingly higher in the BBM compared with the cultured cells. Because differences in the expression of P-gp, MRP5, and MRP6 were observed in the various BBB models using reverse transcription-polymerase chain reaction techniques, the disparity in transport kinetics between the BBB models may be linked to variations in the amount or type of drug efflux transporters expressed in each model. The present study introduces a method of quantitatively evaluating drug efflux transport kinetics in the BBB.

The blood-brain barrier (BBB) presents a formidable challenge to the delivery of therapeutic agents to the central nervous system. The brain microvessel endothelial cells forming the BBB have several morphological and biochemical features that restrict the movement of molecules from the general circulation into the brain (Rubin and Staddon, 1999). These features include tight junctions between the cells, a lack of fenestrations, and low pinocytic activity (Goldstein and Betz, 1986). An additional feature that contributes to the restrictive nature of the BBB is the expression of a variety of drug efflux transport systems.

P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP), and breast cancer resistant protein (BCRP) are drug efflux transporters that use energy provided by ATP hydrolysis to actively move compounds out of the cell (Schinkel and Jonker, 2003). These proteins are expressed in the BBB and are involved in the transport of a wide variety of compounds out of the brain (Sun et al., 2003). For a growing number of therapeutic agents, this translates into limited brain permeability and a lack of therapeutic efficacy. Due to the impact of drug efflux transport on drug disposition in the brain, there has been a concerted effort to design drugs that have minimal interactions with these transport systems as well as to identify modifying agents capable of inhibiting the function of these transporters.

Most of the assays currently used to investigate drug efflux interactions primarily focus on the affinity (K_m) of a drug for a particular drug efflux protein. Less emphasis has been placed on understanding and predicting, in a more quantitative fashion, the capacity of the drug efflux transporters to influence permeability across biological barriers (i.e., V_max and intrinsic clearance). This is especially the case in tissues such as the BBB that express multiple drug efflux transporters. A reliable method for determining the Michaelis-Menten kinetics of various drug efflux transporters in a whole cell preparation would provide an additional tool for predicting the central nervous system disposition of drugs. In addition, although differences in drug efflux transporter expression have been reported in various models of the brain...

ABBREVIATIONS: BBB, blood-brain barrier; P-gp, P-glycoprotein; MRP, multidrug resistance-associated protein; BCRP, breast cancer resistant protein; BBM, bovine brain microvesSEL(s); BBMEC, bovine brain microvesSEL endothelial cell(s); MDCKII-MDR1, Madin-Darby canine kidney epithelial cell(s) transfected with the human MDR1 gene; BCECF-AM, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; 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; BCECF, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; RT-PCR, reverse transcription-polymerase chain reaction; EBG, estradiol-17β-glucuronide.
BBB (Regina et al., 1998; Zhang et al., 2000), the ability to quantitatively determine the kinetic parameters of drug efflux transporter activity would be of use in correlating changes in expression with alterations in transport function.

Previously, our lab characterized the drug efflux interactions of the fluorescent probe BCECF and demonstrated its utility in assessing drug efflux activity in the BBB (Bachmeier et al., 2004, 2005; Bachmeier and Miller, 2005). These studies suggest that BCECF accumulation in brain endothelial cells was dependent on both P-gp and MRP-mediated transport and that the individual contributions of each transporter could be determined with appropriate inhibitors (Bachmeier et al., 2004). The purpose of the present study was to: 1) develop a method for determining the \( K_m \) and \( V_{\text{max}} \) of P-gp and MRP-related transport systems in the BBB using BCECF as the probe, and 2) use this method to quantitatively assess the drug efflux kinetics of various BBB models, including freshly isolated bovine brain microvessels (BBM), primary cultured bovine brain microvessel endothelial cells (BBMEC), and the MDCKII-MDR1 cell line.

The present study demonstrated similarities in the affinity (\( K_m \)) of BCECF-AM for P-gp in all three BBB models examined. However, the \( V_{\text{max}} \) was considerably higher in the BBM compared with the cultured cells. For MRP-related transport, the \( K_m \) of BCECF varied widely with the MDCKII-MDR1 cells demonstrating the highest affinity. As was the case for P-gp, the \( V_{\text{max}} \) of BCECF for MRP-related transport was overwhelmingly higher in the BBM. In addition, the \( V_{\text{max}} \) correlated with the expression levels of the various transporters. Using the BCECF probe, the current study provides a quantitative assessment of the drug efflux kinetics of P-gp and MRP-related transport in various BBB models.

### Materials and Methods

#### Materials/Reagents

BCEF-AM and its free acid (BCEF) were purchased from Molecular Probes (Eugene, OR). Colchicine, indomethacin, bovine fibronectin, and equine serum were purchased from Sigma Chemical Co. (St. Louis, MO). GF120918 was provided by GlaxoSmithKline (Research Triangle Park, NC). Falcon tissue culture plates, minimum essential media, Dulbecco’s modification of Eagle’s medium, Ham’s F-12, Triton-X-100, and rat tail collagen were purchased from Fisher Scientific (St. Louis, MO). Fetal bovine serum, trypsin-EDTA, and penicillin/streptomycin were purchased from Gibco BRL products (Rockville, MD). Bicinchoninic acid protein assay kit was purchased from Pierce (Indianapolis, IN). Madin-Darby canine kidney epithelial cells transfected with the human MDR1 gene (MDCKII-MDR1) were provided by M. M. Gottesman (National Cancer Institute, Bethesda, MD).

#### Cell Isolation and Culturing

BBMEC were isolated from the gray matter of fresh bovine cerebral cortices using enzymatic digestion and centrifugal separation methods as described previously (Miller et al., 1992). Freshly isolated BBM were either used immediately for the drug efflux transporter studies or used to establish primary cultures of BBMEC. For the primary cultures, BBMEC were seeded (50,000 cells/cm\(^2\)) on collagen-coated, fibronectin-treated, 24-well polystyrene tissue culture plates (2 cm\(^2\)/well). The culture media consisted of 45% minimum essential medium, 45% Ham’s F-12 nutrient mix, 10 mM HEPES, 13 mM sodium bicarbonate, 50 \( \mu \)g/mL gentamicin, 10% equine serum, 2.5 \( \mu \)g/mL amphotericin B, and 100 \( \mu \)g/mL heparin. The BBMEC were cultured in a humidified 37°C incubator with 5% CO\(_2\), with medium replacement occurring every other day until the monolayers reached confluence (approximately 3–4 days).

#### Cellular Kinetic Studies

Drug efflux transporter activity was examined by monitoring BCECF accumulation in BBM, BBMEC, and MDCKII-MDR1 cell preparations. Because BCECF accumulation can be influenced by both P-gp and MRP transport systems (Bachmeier et al., 2004), P-gp activity was determined indirectly by taking the difference between BCECF accumulation under normal conditions and after complete pharmacological inhibition of P-gp with GF120918 (3.2 \( \mu \)M). For the kinetic analysis of P-gp, cells were pretreated in pH 7.4 Tyrode’s balanced salt solution (TBSS; 136 mM NaCl, 2.6 mM KCl, 1.8 mM CaCl\(_2\), 1 mM MgCl\(_2\), 0.36 mM NaH\(_2\)PO\(_4\), 5.56 mM D-glucose, and 5 mM HEPES) in the presence and absence of GF120918 (3.2 \( \mu \)M) for 30 min at 37°C. After the pretreatment period, solutions were removed, and the cells were incubated at 37°C in Tyrode’s balanced salt solution containing various concentrations of BCECF-AM (0.032–10 \( \mu \)M) for 30 min in the presence and absence of the P-gp/BCRP inhibitor GF120918. After the 30-min exposure to BCECF-AM, control and GF120918-treated cells were washed with ice-cold phosphate-buffered saline and solubilized in 1% Triton X-100 for the determination of intracellular BCECF (\( \lambda_{ex} = 505 \text{ nm} \) and \( \lambda_{em} = 535 \text{ nm} \)) using a fluorescence spectrophotofluorometer (Shimadzu RF5000; Shimadzu, Kyoto, Japan).

In determining MRP-related transport kinetics, the various cell preparations were preincubated in buffer solution in the presence of GF120918 (3.2 \( \mu \)M). The solutions were removed and the cells exposed to BCECF-AM (0.032–10 \( \mu \)M) for 30 min in the presence of GF120918. At the end of the 30-min treatment period, the buffer solution was collected to determine extracellular BCECF concentrations. The cells were then washed and solubilized as described above to determine intracellular BCECF concentrations. Transport through MRP-mediated processes was based on the amount of BCECF effluxed from the intracellular compartment to the extracellular compartment over the 30-min treatment period. The protein content in the samples was determined using the Pierce bicinchoninic acid method.

#### Establishment of Kinetic Model and Parameters

Drug efflux transport kinetics were based on the cellular compartmental model shown in Fig. 1. To examine drug efflux transport, the cells were exposed to BCECF-AM. The ester-based BCECF-AM readily diffuses into the cell where it is then rapidly hydrolyzed to the free acid form BCECF. Whereas BCECF-AM is nonfluorescent, the free acid form is readily fluorescent at excitation and emission wavelengths of 505 and 535 nm, respectively. Based on previous studies, the cell-permeable ester form of BCECF (BCEF-AM) is a substrate for P-gp-mediated transport, whereas the nonpermeable free acid (BCEF) is a substrate for MRP-related transport (Bachmeier et al., 2004).

Because BCECF-AM is nonfluorescent, assessment of P-gp activity was determined by calculating the difference between the intracellular accumulation of BCECF under control conditions and after complete pharmacological inhibition of P-gp (i.e., in the presence of GF120918). Using this method and the compartmental model shown in Fig. 1, the rate of change in intracellular BCECF under control conditions is represented by \( q_1 \), whereas the rate of change in intracellular BCECF after complete inhibition of P-gp by GF120918 is represented by \( q_2 \). The difference between eqs. 1 and 2 represents the rate of P-gp transport of BCECF-AM (eq. 3).

![Fig. 1. Compartmental schematic describing the cellular drug efflux transport kinetics of BCECF-AM and its metabolite BCECF. Compartment 1 represents extracellular BCECF-AM, compartment 2 represents intracellular BCECF, and compartment 3 represents extracellular BCECF. The rate constants for simple diffusion, P-gp, and MRP-related transport are indicated by \( k_{12} \), \( k_{21} \), and \( k_{23} \), respectively.](image-url)
The MRP-related drug efflux kinetics of BCECF were determined directly by measuring the amount of BCECF transported into the extracellular environment over time as represented by eq. 4.

\[
\frac{dA_{2}(t)}{dt} = k_{12}A_{1} - k_{23}A_{2}
\]

The rate constants for simple diffusion, P-gp transport, and MRP-related transport are indicated by \( k_{12} \), \( k_{21} \), and \( k_{23} \), respectively. \( A_{1} \) represents the amount of extracellular BCECF in compartment 1, \( A_{2} \) represents the amount of intracellular BCECF in compartment 2 under control conditions, and \( k_{23} \) represents the amount of intracellular BCECF in compartment 2 in the presence of GF120918, and \( A_{3} \) represents the amount of extracellular BCECF in compartment 3. The data were plotted as velocity divided by concentration versus velocity and fitted to acquire the kinetic parameters for P-gp or MRP-related transport.

Because the P-gp kinetics with BCECF-AM were determined indirectly through the accumulation of the probe in the cell, the actual \( K_{m} \) was calculated by multiplying the observed \( K_{m} \) by a correction factor. This correction was necessary because the observed \( K_{m} \) is based on the concentration of BCECF-AM exposed to the cells (i.e., the extracellular concentration). However, the extracellular concentration of BCECF-AM does not represent the level of probe that is actually available to the cell for transport out. To establish the actual amount available for P-gp-mediated efflux transport, it was necessary to determine the fraction of extracellular BCECF-AM that made it into the cell (i.e., the correction factor). This was done by measuring the amount of BCECF inside the cell after P-gp inhibition and dividing it by the amount of BCECF in the extracellular compartment. The original \( K_{m} \) was then multiplied by the correction factor to give the actual \( K_{m} \) value. Without this correction, the original \( K_{m} \) values would underestimate the affinity of the probe for P-gp.

Assumptions of Kinetic Model. In using the current methods to evaluate drug efflux kinetics, a number of assumptions must be made. The first assumption is that diffusion of BCECF-AM out of the cell is minimal. It is known that BCECF-AM enters the cell through simple diffusion. Although it may exit the cell in the same manner, any back flux seems to be minimal or nonexistent because of the rapid rate of hydrolysis of BCECF-AM to BCECF that occurs inside the cell. This is based on prior work in our lab that demonstrated intracellular BCECF-AM was not detectable at any of the time points examined, indicating immediate conversion to BCECF once inside the cell (data not shown). For this reason, movement of BCECF-AM out of the cell through simple diffusion was not included in the schematic in Fig. 1 or factored into the kinetic equations used in the present study. Likewise, because of the anionic nature of BCECF, it is unlikely that this metabolite will passively diffuse into or out of the cell to any appreciable extent. This forms the basis for the second assumption, which is the appearance of BCECF in the extracellular environment is the result of intracellular metabolism of BCECF-AM and the subsequent transport of BCECF out of the cell. In other words, the appearance of BCECF in the extracellular solution is not due to hydrolysis of BCECF-AM before entry into the cell. The third assumption is that the concentration of GF120918 used in the present study is able to completely inhibit P-gp transport. If P-gp function were not completely inhibited, the subsequent results would underestimate the transport capacity of P-gp. Evidence in support of this assumption is based on previous concentration-response curves generated in BBM monolayers demonstrating maximal inhibition of rhodamine 123 efflux from the cells with concentrations of GF120918 greater than 1 \( \mu M \) (Bachmeier and Miller, 2005). This is consistent with the maximal inhibitory concentrations of GF120918 for P-gp found in the literature (Hyafil et al., 1993; Wallstab et al., 1999; Chen et al., 2000). The final assumption is that enzyme metabolism is not the rate-limiting step in the elimination of BCECF. Given the rapid and extensive conversion to BCECF inside the cell it is assumed that MRP-related transport is the rate-limiting step in the elimination of BCECF, not metabolism.

RNA Isolation and RT-PCR. Total RNA was isolated from the BBM, MDCKII-MDR1 cells, and freshly isolated BBM cells as described previously (Hui-Yun et al., 1998). Single-strand cDNA was synthesized from 1 \( \mu g \) of RNA by reverse transcription using random hexamers (Perkin Elmer, Branchburg, NJ). The cDNA solution was amplified with primers previously reported for MRP1 (Hui-Yun et al., 1998), MRP2–MRP6 (Kool et al., 1997, 1999), and MDR1 (Sobue et al., 1999). The reaction conditions were carried out in the same manner as described previously (Zhang et al., 2000). Samples of each single-strand cDNA were also treated with primers that amplify a 661-base pair fragment of the \( \beta \)-actin gene (Stratagene, La Jolla, CA) to allow for quantitative comparison with the desired gene samples. The products from the PCR amplification were separated on 2% agarose gel and visualized with ethidium bromide.

Results

P-gp Kinetic Studies. Representative plots of the transport of BCECF-AM by P-gp and BCECF by MRP-related proteins are displayed in Fig. 2 and Fig. 3, respectively. For BCECF-AM, the \( K_{m} \), \( V_{max} \), and \( V_{max}/K_{m} \) (intrinsic clearance) for P-gp was 1.58 \( \mu M \), 48 pmol/min \( \cdot \) mg protein, and 32 \( \mu l/min \cdot mg\ protein \), respectively, in the MDCKII-MDR1 cells (Table 1). In the BBM, \( K_{m} \), \( V_{max} \), and \( V_{max}/K_{m} \) values were larger that observed in MDCKII-MDR1 cells (2.78 \( \mu M \) and 100 pmol/min \( \cdot \) mg protein, respectively), although the intrinsic clearance (\( V_{max}/K_{m} \)) was essentially the same in both cell culture models (37 \( \mu l/min \cdot mg\ protein \)). In the BBM, the \( K_{m} \) for P-gp was approximately the same as in the BBMEC (2.68 \( \mu M \). However, the \( V_{max} \) and \( V_{max}/K_{m} \) in the BBM preparations were over 4-fold greater than either the MDCKII-MDR1 or BBMEC culture models (428 pmol/min \( \cdot \) mg protein and 202 \( \mu l/min \cdot mg\ protein \), respectively) (Table 1).

MRP Kinetic Studies. With respect to BCECF and MRP-related transport in the MDCKII-MDR1 cells, the \( K_{m} \), \( V_{max} \), and \( V_{max}/K_{m} \) were 178 \( \mu M \), 92 pmol/min \( \cdot \) mg protein, and 0.5209 \( \mu l/min \cdot mg\ protein \), respectively (Table 2). In the BBM, both the \( K_{m} \) and \( V_{max} \) were considerably larger (474 \( \mu M \) and 122 pmol/min \( \cdot \) mg protein, respectively), whereas the \( V_{max}/K_{m} \) was half that observed in the MDCKII-MDR1 cells (0.2623 \( \mu l/min \cdot mg\ protein \)) (Table 2). The kinetics of BCECF were also observed after exposure to a competitive inhibitor. In the presence of indomethacin (32 \( \mu M \)), the \( K_{m} \) in the BBM increased 2.75-fold, whereas less of a change was observed in \( V_{max} \) (1.5-fold) compared with untreated BBM monolayers. In

\[
\frac{dA_{2}(t)}{dt} = k_{12}A_{1} - k_{23}A_{2}
\]
FIG. 3. Representative Eadie-Hofstee plots of the MRP-related transport of BCECF in freshly isolated BBM, primary cultured BBMEC, and MDCKII-MDR1 cells. Values are based on triplicate samples for each preparation.

the BBM, all three parameters were larger for MRP-related transport (1448 μM, 624 pmol/min · mg protein, and 0.4473 μM/min · mg protein for \( K_m \), \( V_{max} \), and \( V_{max}/K_m \), respectively) (Table 2).

**Discussion**

Drug efflux transport is an essential component of the BBB and plays an important role in the brain disposition of a wide variety of drugs. Therapeutic agents interacting with these transporters are likely to have a limited permeability and/or a rapid elimination from the brain. The ability to quantitate the kinetics of these transporters would greatly advance the understanding of their contribution to the BBB. Furthermore, with a quantitative measurement of transporter activity, comparisons of drug efflux activity between various BBB models can be made to identify those models that most accurately reflect the drug efflux characteristics of the BBB in vivo. The current study describes a method for obtaining and evaluating the transport kinetics of P-gp and the MRP-related proteins expressed in various models of the BBB.

Regarding P-gp transport kinetics, the affinities (\( K_m \)) of BCECF-AM for P-gp in all three BBB models were quite similar. It is not surprising that the bovine BBB models displayed nearly identical \( K_m \) values because they are of the same species and tissue origin. The comparable \( K_m \) value in the MDCKII-MDR1 cells indicates the affinity of BCECF-AM for human P-gp is similar. Species differences with regard to P-gp substrates, especially between mouse and human P-gp, have been reported (Schinkel et al., 1996; Schwab et al., 2003). Studies by Schinkel et al. (1996) showed a slight to moderate bidirectional permeability difference in \( mdr1a \) (mouse)-transfected cells with haloperidol, phenytoin, and ondansetron compared with \( MDR1 \) (human)-transfected cells (Schwab et al., 2003). More recent studies suggest some of the apparent species variability between mouse and human P-gp may be related to the absence of the second gene encoding P-gp in the mouse, \( mdr1b \), in many of the screening assays (Schwab et al., 2003).

More importantly, from a methodology standpoint, the \( K_m \) values determined for BCECF-AM and P-gp transport in the present study are similar to the \( K_m \) value of BCECF-AM obtained previously using a P-gp ATPase assay (Homolya et al., 1993). In these studies, the \( K_m \) value was reported to be 1.0 μM (Homolya et al., 1993). The similarity of the \( K_m \) values in the present study with those reported in the literature further validates the current method used for determining P-gp drug efflux transporter kinetics.

Although the \( K_m \) values for P-gp in all three BBB models were similar, there were significant variations in the \( V_{max} \) values determined for each of the BBB models, with the freshly isolated BBM displaying a considerably larger \( V_{max} \) for P-gp than either the cultured BBMEC or MDCKII-MDR1 cells. The rank order was BBM > BBMEC > MDCKII-MDR1. The differences in \( V_{max} \) in the various BBB models is not surprising that the bovine BBB models displayed nearly identical \( K_m \) values because they are of the same species and tissue origin. The comparable \( K_m \) value in the MDCKII-MDR1 cells indicates the affinity of BCECF-AM for human P-gp is similar. Species differences with regard to P-gp substrates, especially between mouse and human P-gp, have been reported (Schinkel et al., 1996; Schwab et al., 2003).

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**TABLE 1**

<table>
<thead>
<tr>
<th>BBB Model</th>
<th>( K_m ) (μM)</th>
<th>( V_{max} ) (pmol/min · mg protein(^{-1}))</th>
<th>( V_{max}/K_m ) (μM/min · mg protein(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBM</td>
<td>2.68 ± 0.83</td>
<td>428.9 ± 85.6</td>
<td>202.4 ± 69.6</td>
</tr>
<tr>
<td>MDCKII-MDR1</td>
<td>1.58 ± 0.26</td>
<td>48.2 ± 20.6</td>
<td>32.6 ± 12.9</td>
</tr>
<tr>
<td>BBMEC</td>
<td>2.78 ± 0.36</td>
<td>100.2 ± 5.8</td>
<td>36.9 ± 6.8</td>
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</tbody>
</table>

Values represent mean ± S.E.M. (\( n = 9 \)) from three separate studies.

**TABLE 2**

<table>
<thead>
<tr>
<th>BBB Model</th>
<th>( K_m ) (μM)</th>
<th>( V_{max} ) (pmol/min · mg protein(^{-1}))</th>
<th>( V_{max}/K_m ) (μM/min · mg protein(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBM</td>
<td>1448.2 ± 183.1</td>
<td>624.9 ± 38.2</td>
<td>0.447 ± 0.083</td>
</tr>
<tr>
<td>MDCKII-MDR1</td>
<td>178.4 ± 12.4</td>
<td>92.5 ± 3.6</td>
<td>0.521 ± 0.023</td>
</tr>
<tr>
<td>BBMEC</td>
<td>474.3 ± 98.1</td>
<td>122.8 ± 22.9</td>
<td>0.262 ± 0.015</td>
</tr>
<tr>
<td>BBMEC + indomethacin</td>
<td>1299.6 ± 94.2</td>
<td>181.1 ± 4.5</td>
<td>0.141 ± 0.014</td>
</tr>
</tbody>
</table>

Values represent mean ± S.E.M. (\( n = 9 \)) from three separate studies.
BBB models could reflect alterations in P-gp expression. In support of this, RT-PCR studies showed an approximately 2-fold increase in P-gp at the mRNA level in freshly isolated BBM compared with primary cultured BBMEC. Whereas differences in P-gp expression in the three BBB models would not be expected to affect $K_m$, such differences would influence the observed $V_{max}$ and consequently intrinsic clearance values for BCECF-AM in the three models.

The $V_{max}$ and intrinsic clearance data obtained in the present study for P-gp using BCECF-AM correlate well with previous studies examining differences in P-gp expression in various BBB models (Barrand et al., 1995; Regina et al., 1998; Zhang et al., 2000). Barrand et al. (1995) noted a significant reduction in P-glycoprotein expression in cultured brain endothelial cells compared with freshly isolated brain capillaries. Studies by Regina et al. (1998) found P-gp expression to be greatest in freshly isolated brain microvessels and lowest in immortalized brain microvessel endothelial cells, with the primary brain endothelial cell culture model having an intermediate level of expression. Thus, the higher $V_{max}$ and intrinsic clearance values for P-gp function obtained in the present study with freshly isolated BBM compared with the cell culture models of the BBB are probably attributable to decreased P-gp expression in the cultured cells.

Although the method for acquiring the transport kinetics of MRP-related activity is fairly straightforward, interpretation of the results is more complex than for P-glycoprotein. The major complication involves the presence of not one type of protein but several isoforms of MRP and potentially other organic anion transporters (e.g., OAT and OATP) in the BBB (Zhang et al., 2000; Kusuhara and Sugiyama, 2001, 2002). It is anticipated that BCECF interacts with most, if not all, of the MRP transporters expressed in the BBB. As a result, it is not possible at the current time to identify the contributions of individual MRP efflux proteins. Therefore, the $K_m$ value of BCECF for MRP-related transport determined in the present study represents an average $K_m$ for all of the efflux transporters that BCECF interacts with. In the context of this study, $V_{max}$ represents the cumulative effect of all the efflux transporters participating in the elimination of BCECF. Although a composite kinetic profile of drug efflux transporter activity is more difficult to interpret, it does reflect the functional reality present in many cellular barriers and elimination organs, where multiple transport processes are determining distribution and elimination kinetics. Recent studies by Shilling et al. (2005) using isolated canaliculur membrane vesicles examined the transport kinetics of leukotriene C4 and estradiol-17β-β-glucuronide (EBG). These studies report both high-affinity (leukotriene C4) and low-affinity (EBG) clearance in the isolated canaliculur membrane vesicles. The large $K_m$ values for EBG (>$300 \mu M$) were attributed to multiple drug efflux transporter interactions and potential cooperativity between drug efflux transport systems (Shilling et al., 2005).

In contrast to the P-gp-related kinetics that had relatively consistent $K_m$ and variable $V_{max}$ values in the three BBB models examined, both $K_m$ and $V_{max}$ values for MRP-related transport varied considerably among the models examined. The affinity ($K_m$) of BCECF for MRP-related transport was highest in the MDCKII-MDR1 cells, whereas the BBB model that displayed the lowest $K_m$ for MRP-related transport was the freshly isolated BBM. Conversely, $V_{max}$ values for BCECF transport were greatest in the BBM, whereas the BBMEC and MDCKII-MDR1 had approximately 6-fold lower values. Because
BCECF is a substrate for multiple organic anion transporters, the dramatic differences in $K_m$ and $V_{max}$ values determined for the various BBB models may be due to variations in the profile of MRP-related transporters expressed in each of the three models. Previous studies have demonstrated differences in MR expression between cultured BBMCEC and freshly isolated brain capillaries (Regina et al., 1998; Zhang et al., 2000). Studies by Regina et al. (1998) reported increased MRP1 protein expression in primary brain endothelial cell cultures compared with freshly isolated brain microvessels. Comprehensive studies by Zhang et al. (2000) examined the RNA expression profile for MRP1–MRP6 in freshly isolated BBM and primary cultured BBMCEC. Whereas the level of expression of most of the individual homologs were similar, there was an increased expression of MRP5 and a decreased expression of MRP6 in BBM compared with the primary cultured BBMCEC (Zhang et al., 2000). This was confirmed in the present study where the freshly isolated BBM expressed MRP5, but not MRP6, whereas the BBMCEC expressed MRP6 and little or no MRP5. The MDCCKII-MDR1 cells were similar to the BBMCEC because they too expressed some MRP6 and had no detectable MRP5. These differences in MR expression between the three models examined could contribute greatly to the variations in $K_m$ and $V_{max}$ for BCECF observed in the present study.

Although the potential involvement of multiple efflux transport systems in the elimination of BCECF from the various BBB models can complicate interpretation of the kinetic data, a potentially useful feature of the present MR-related assay is the ability to mechanistically evaluate the effect of various inhibitors on the kinetics of MR-related transport to determine either the type of inhibition or the influence of individual drug efflux transporters. In the present study, the application of indomethacin produced a substantial increase in $K_m$ and only slightly altered the observed $V_{max}$. These changes are indicative of a competitive type of inhibition by indomethacin. These findings are supported by Draper et al. (1997), who suggested indomethacin modulates drug efflux through direct competition at the transport site. As more specific MRP inhibitors become available, either through small molecule drug discovery or siRNA technology, the current technique is well positioned to examine the contribution of individual MRP transport systems in the BBB.

Using the fluorescent probe BCECF, the present study provides a quantitative comparison of the drug efflux kinetics of P-gp and MRP-related transport in various models of the BBB. Of the three models examined, BBM had the highest capacity and greatest intrinsic clearance for P-gp-mediated transport compared with the BBMCEC and MDCKII-MDR1 culture models. For MRP-related transport, there were substantial differences in both $V_{max}$ and $K_m$ for BCECF transport in all three models examined. From a more general perspective, the current study describes a method for performing quantitative Michaelis-Menten kinetics on drug efflux transporters in whole cell preparations. As such, this approach could be very beneficial as an additional tool for characterizing drug efflux transporter interactions of compounds as well as quantitative determination of the impact of mutations and polymorphism on drug efflux transporter function.

References


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