Lack of P-Glycoprotein-Mediated Efflux and the Potential Involvement of an Influx Transport Process Contributing to the Intestinal Uptake of Deltamethrin, cis-Permethrin, and trans-Permethrin

Jason Zastre,¹ Chris Dowd, James Bruckner, and Andrew Popovici

Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, University of Georgia, Athens, GA 30602

¹To whom correspondence should be addressed at Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, University of Georgia, 250 west Green Street, Athens, GA 30602. Fax: (706) 542-5358. E-mail: jzastre@rx.uga.edu.

Received May 3, 2013; accepted August 9, 2013

The effectiveness and widespread use of pyrethroid insecticides has lead to concerns regarding their safety. Human ingestion of these potentially neurotoxic compounds is typically through hand-to-mouth contact or consumption of contaminated foods. A substantial proportion of ingested pyrethroids are eliminated in feces, suggesting that absorption is limited, possibly by the action of the efflux transporter P-glycoprotein (P-gp). We utilized caco-2 cells as a model system for intestinal enterocytes and qualitatively and quantitatively assessed the transport of deltamethrin (DLM), cis-permethrin (CPM), and trans-permethrin (TPM). Caco-2 cell uptake of the P-gp substrate R6G was increased by the P-gp inhibitors cyclosporine A (CSA) and ritonavir but not by DLM, CPM, and TPM. Unexpectedly, CSA and ritonavir significantly reduced the uptake of DLM, CPM, and TPM. Permeability coefficients (P_app) and directional flux of DLM, CPM, or TPM were greater in the absorptive than the secretory (efflux) direction when measured across caco-2 monolayers grown on Transwell inserts. When CSA was applied to the monolayers’ apical (AP) side, the AP to basolateral (BL) P_app was significantly reduced, with no change in the BL to AP P_app. Kinetic analysis demonstrated saturable transport kinetics for all 3 pyrethroids. These findings indicate that the cellular uptake of DLM, CPM, and TPM is not limited by P-gp efflux but undergo absorptive influx transport as a contributing mechanism for cellular uptake. However, the overall P_app values for DLM, CPM, and TPM are consistent with the low permeability/low absorption compound mannitol, suggesting limited gastrointestinal absorption potential.

Key Words: P-glycoprotein; pyrethroid; insecticide; transport; caco-2.

The sale of pyrethroid insecticides has increased dramatically in the United States and Europe, with the decline in utilization of organophosphates (Power and Sudakin, 2007). Pyrethroids are used agriculturally to protect a wide variety of crops. Thus, these insecticides are frequently detected in relatively low concentrations in some fruits and vegetables (Lu et al., 2010). Certain pyrethroids are also utilized on livestock, in food storage and transportation, and in indoor and outdoor residential settings. About 70% of permethrin (PM), commonly a mixture of its cis-permethrin (CPM) and trans-permethrin (TPM) isomers, is employed in nonagricultural settings, including treating pets and children as a pediculicide for head lice (Naeher et al., 2009).

Pyrethroids exert neurotoxicity in mammals and insects by slowing the opening and closing of voltage-gated sodium channels in the axons of the peripheral and central nervous systems (Soderlund, 2012). The sequences of neurological events and manifestations of toxicity depend upon the compounds’ structure. Type I pyrethroids, such as PM and bifenthrin, do not contain a cyano group. Tremors and paresthesias are their major signs of poisonings. Type II pyrethroids, such as deltamethrin (DLM) and cypermethrin, induce salivation, hypexcitability and choreoathetosis (Soderlund, 2012). There is substantial evidence that the parent pyrethroids are the proximate neurotoxicants. Large segments of the population in the United States and Europe are exposed to pyrethroids, albeit at quite low levels. Pyrethroid metabolites were found in 75% of an urban German population without occupational contact (Heudorf et al., 2004). Similar findings were more recently reported in the general U.S. populace (Barr et al., 2010). Small amounts of pyrethroids are detected in some agricultural commodities. Researchers assessed the relative contributions of exposure pathways of preschool children to CPM and TPM in their homes and daycare centers (Morgan et al., 2007). The primary route of exposure was dietary ingestion, followed by indirect (hand-to-mouth) ingestion.

Remarkably little is known about the gastrointestinal (GI) absorption of pyrethroids. They might be anticipated to readily pass through GI mucosal cells by passive diffusion, as these compounds are very lipophilic. In vivo studies in adult rats, however, have shown the oral bioavailability of DLM to range...
from just 18% to 28% (Godin et al., 2010; Kim et al., 2008). Bioavailability may not be an accurate measure of oral absorption, as compounds absorbed into the venous system will be subject to first-pass hepatic metabolism. DLM is extensively metabolized by CYP450s and carboxylesterases (Anand et al., 2006). Abraham et al. (1994) monitored systemic uptake and fecal excretion of several polyaromatic hydrocarbons in a breast-fed infant. Fecal excretion, apparently due to incomplete systemic uptake, was greater for the more highly chlorinated (ie, more lipophilic) chemicals. Ruzo et al. (1978) found 36% of a 0.9-mg/kg oral dose of DLM in the feces of rats. Brain DLM levels were less than 20% of blood levels in orally dosed rats, an unexpected finding for the highly lipophilic compound. P-glycoprotein (P-gp), a membrane efflux transporter for a wide variety of substrates, is expressed in many cells/tissues including hepatocytes, blood-brain barrier, and GI epithelium (Abu-Qare et al., 2003). P-gp might, therefore, contribute to these findings.

Caco-2 cells have been widely used and accepted by the pharmaceutical industry and regulatory authorities for assessing intestinal permeability (Shah et al., 2006). Caco-2 cells, a well-characterized human colon adenocarcinoma cell line, can be cultured on dishes or permeable membranes (Transwell), providing discrete apical (AP) and basolateral (BL) sides. These cells form a confluent monolayer of polarized columnar cells with tight junctions and microvilli on their AP membrane. The majority of intestinal enzymes and transporters are expressed (Sun et al., 2008). Caco-2 cell permeability frequently correlates well with in vivo absorption in humans (Artursson and Karlsson, 1991). With caco-2 cells, it is possible to learn whether a drug is actively or passively transported across intestinal epithelium, as well as to discern efflux from influx (Artursson et al., 2001). There have been reports that several pyrethroids bind to P-gp (Bain and LeBlanc, 1996; Pivčević and Zaja, 2006; Sreeramulu et al., 2007). However, no investigators to our knowledge have assessed the ability of P-gp to transport them in intestinal enterocytes.

The overall objective of the current investigation was to characterize the GI absorption of 3 common pyrethroids (DLM, CPM, and TPM) in vitro. An important goal was to learn whether pyrethroids are absorbed passively or actively and whether they are substrates for efflux or influx transporters in enterocytes. Caco-2 cell monolayers and P-gp overexpressing cells were utilized to test the hypothesis that P-gp-mediated efflux transport of pyrethroids contributes to their limited oral absorption.

**MATERIALS AND METHODS**

**Materials.** Cell culture media and supplements, which include Dulbecco’s modified eagle’s medium (DMEM), nonessential amino acids (NEAA), t-glutamine, penicillin/streptomycin, and trypsin/EDTA were obtained from Mediatech (Manassas, Virginia). Fetal bovine serum (FBS) and human serum albumin (HSA) were purchased from Sigma-Aldrich (St. Louis, Missouri). Cell culture–treated flasks, plates, and dishes were from Greiner Bio-one (Monroe, North Carolina). Cyclosporin A (CSA) was purchased from Amresco (Salon, Ohio), and ritonavir was obtained from Yes Pharma (Beit Shemesh, Israel). Rhodamine 6G (R6G) was obtained from Emd Chemicals (Gibbstown, New Jersey). DLM was provided by Bayer CropScience (Kansas City, Missouri), and CPM and TPM were a gift from FMC Co (Philadelphia, Pennsylvania). Radiolabeled [14C]-DLM (54.1 mCi/mmol) was supplied by Bayer CropScience (Stilwell, Kansas). [14C]-CPM (61 mCi/mmole) and [14C]-TPM (61 mCi/mmole) were provided by Symbiotic Research (Mount Olive, New Jersey). Radiolabeled [3H]-mannitol (20 Ci/mmole) and [3H]-propanolol (23.2 Ci/mmole) were purchased from American Radiolabeled Chemicals (St. Louis, Missouri).

**Cell culture.** Caco-2 cells were obtained from ATCC (Rockville, Maryland) as passage 17 and cultured as previously described (Zastre et al., 2004). Briefly, caco-2 cells were grown in a humidified incubator at 37°C under 5% CO2 using DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 1% t-glutamine, and 1% NEAA. Before reaching confluency, cells were trypsinized and seeded into either 24-well plates or 0.4-μm polycarbonate membrane Transwell inserts (Corning, Lowell, Massachusetts) at a density of 40,000 cells/cm2. Cell media was changed every 2–3 days, and the cells used for experiments between 21 and 28 days postseeding for transport or uptake experiments. Monolayer integrity was routinely assessed by quantifying the rate of mannitol flux, with monolayers having < 0.25%/h/cm2 only used in experiments (Walle and Walle, 1998). The P-gp overexpressing cell line MDCK-MDR1 and wild-type MDCK cells were kindly donated by Dr Helen Burt (Wan et al., 2013).

**Uptake experiments.** Cellular accumulation of the P-gp substrate R6G, DLM, CPM, and TPM by caco-2, MDCK, and MDCK-MDR1 cells with and without the P-gp inhibitors CSA (25μM) and ritonavir (50μM) was assessed at 37°C for 3 min. The concentrations of inhibitors were chosen because they maximally increased R6G and decreased DLM, CPM, and TPM uptake (data not shown). Prior to accumulation experiments, the cells were washed twice with 37°C Hanks balanced salt solution (Mediatech, Manassas, Virginia) containing 10mM hepes pH = 7.2 (hereafter, referred to as transport buffer). Cells were then preincubated for 30 min with transport buffer with or without inhibitor, 0.5μM R6G, 1μM [14C]-DLM, [14C]-CPM, or [14C]-TPM in transport buffer was added with or without CSA or ritonavir. A similar protocol was used to establish whether DLM, CPM, or TPM can inhibit P-gp-mediated efflux of R6G. Briefly, 0.5μM R6G with or without 10μM of DLM, CPM, or TPM was incubated at 37°C for 3 min. After this uptake time, wells were washed with ice-cold PBS and lysed with 1% Triton X-100. An aliquot was measured using liquid scintillation for quantitation of DLM, CPM, and TPM, and the results were normalized to total protein (BCA protein assay kit, Thermo Scientific, Rockford, Illinois). The fluorescence intensity of R6G in cell lysates was measured using a SpectraMax M2E (Molecular Devices, Sunnyvale, California) with λex = 508 nm and λem = 560 nm and normalized to total protein.

**Directional flux experiments.** The flux of R6G, DLM, CPM, and TPM across caco-2 monolayers was determined in the absorptive AP to BL and secretory BL to AP directions (Fig. 1). Caco-2 cells grown on Transwell inserts as described above were washed and allowed to equilibrate at 37°C for 15 min with transport buffer.

For AP to BL flux, 0.5 ml of either 0.5μM R6G, 1μM [14C]-DLM, [14C]-CPM, or [14C]-TPM in transport buffer was placed on the AP side and 1.5 ml of transport buffer + 2% HSA placed on the BL side. The inclusion of HSA on the receiver side provided a suitable sink for lipophilic and highly protein-bound compounds. The plate was placed on an incubating orbital shaker (Henry Trowmer LLC, Thorofare, New Jersey) set to 50rpm at 37°C. At specified time points, 3 x 250 μl aliquots were removed from the BL receiver chamber and replaced with an equivalent volume of prewarmed transport buffer + 2% HSA. For BL to AP flux, 1.5 ml of either 0.5μM R6G, 1μM [14C]-DLM, [14C]-CPM, or [14C]-TPM in transport buffer was placed into the BL chamber and 0.5 ml of transport buffer + 2% HSA placed on the AP side. Three aliquots of 50 μl were taken from the AP side at each time point and replaced with an equivalent volume of prewarmed transport buffer + 2% HSA.
Flux of the high-permeability marker propranolol (4nM) and low-permeability marker mannitol (5nM) and directional flux of R6G, DLM, CPM, and TPM in the presence of the P-gp inhibitor CSA (25μM) were performed as described above. Briefly, caco-2 cells were pretreated with CSA in transport buffer applied to the AP side for 30 min. For AP to BL flux, CSA was added with R6G, DLM, CPM, or TPM on the AP side. For BL to AP flux, CSA was added to the transport buffer +2% HSA on the AP receiver side.

The flux of DLM, CPM, TPM, mannitol, and propranolol across caco-2 cells was quantified by liquid scintillation counting using a Beckman Coulter LS 6500 (Brea, California). For R6G, the fluorescence intensity was measured using a SpectraMax M2E (Molecular Devices, Sunnyvale, California) with λex = 508 nm and λem = 560 nm. The apparent permeability coefficient (Papp) was calculated using the following equation:

\[ P_{\text{app}} = \frac{\Delta Q}{\Delta t \cdot A \cdot Co} \]  

Where \( \Delta Q \) is the amount of compound transported (ie, present in the receiving chamber) during the time interval \( \Delta t \). Co is the concentration applied to the donor side/chamber, and \( A \) is the monolayer surface area.

**Kinetic analysis of DLM, CPM, and TPM transport.** To establish an estimation of the Michaelis–Menten constant (\( K_m \)) and maximal transport velocity (\( V_{\text{max}} \)) of DLM, CPM, and TPM, the concentration-dependent uptake by caco-2 cells was performed. The uptake (3 min) of increasing concentrations of \([^{14}C]\)-DLM, \([^{14}C]\)-CPM, or \([^{14}C]\)-TPM in transport buffer was performed as described above. The total uptake rate into caco-2 cells is the sum of all saturable (specific; nonlinear) and nonsaturable (nonspecific; linear) components. The nonsaturable contribution was assessed by the inclusion of CSA (25μM) in the transport of increasing concentrations of DLM, CPM, and TPM. A kinetic analysis was determined by applying a one-site Michaelis–Menten equation with a nonsaturable component using Graphpad Prism 6 software.

\[ V = \frac{V_{\text{max}} [S]}{K_m + [S]} + k_n [S] \]  

Where \( V \) is the total rate of uptake, \( V_{\text{max}} \) is the maximum uptake rate, \( K_m \) is the dissociation constant, [S] is the substrate concentration, and \( k_n \) is the coefficient for nonspecific uptake by diffusion.

**Statistical analysis.** All experiments were performed with a minimum of 3 independent experiments unless otherwise stated. Statistical significance was evaluated between groups using an unpaired Student’s t test with a significance level of \( p < .05 \), using Graphpad Prism 6 software.

**FIG. 1.** Schematic representation of the caco-2 Transwell assay system. Cells form a continuous monolayer over the surface of a porous membrane providing discrete AP and BL chambers. For absorptive flux (AP to BL), compounds are added to the AP chamber and measured as they appear in the BL chamber. For secretory flux (BL to AP), compounds are placed into the BL chamber and monitored as they appear in the AP chamber. Abbreviations: AP, apical; BL, basolateral.

**RESULTS**

**P-gp Activity in Caco-2 Cells**

To confirm functional activity of P-gp in our caco-2 model system, we performed cellular accumulation and directional flux studies with the known P-gp substrate R6G. Figure 2A demonstrates a significant increase in the cellular accumulation of R6G compared to the R6G-only condition. Statistical analysis showed a significant increase in cellular accumulation when CSA (25μM) was added (p < .05).

**FIG. 2.** Functional assessment of P-gp in caco-2 cells was performed using cellular accumulation and flux measurements. A, Cellular accumulation of R6G (0.5μM) with or without the P-gp inhibitors CSA (25μM) or ritonavir (50μM) was assessed at 37°C for 3 min. Results are reported as the mean relative fluorescence (RFU) ± SE normalized to total protein with \( N = 3 \) independent experiments. (*) Statistically significant (\( p < .05 \)) differences between R6G alone to R6G with both inhibitors. B, Flux of 0.5μM R6G across caco-2 monolayers grown on Transwell inserts. R6G AP to BL flux measured the amount of R6G appearing in the BL solution when R6G was placed on the AP side in the presence of (•) 25μM CSA or (*) R6G alone. The BL to AP flux of R6G was determined by placing R6G on the BL side and monitoring the amount that entered the AP side in the presence of (○) 25μM CSA or (■) R6G alone. Data are expressed as the cumulative amount of R6G transported across a unit area of caco-2 monolayers and represented as the mean ± SE with \( N = 3 \). Abbreviations: CPM, cis-permethrin; CSA, cyclosporine A; DLM, deltamethrin; TPM, trans-permethrin.
of R6G in the presence of the P-gp inhibitors CSA and ritonavir. It is apparent that directional flux of R6G in the absorptive (AP to BL) direction was substantially lower than in the secretory (BL to AP) direction (Fig. 2B). The addition of the P-gp inhibitor CSA reduced the BL to AP directional flux to a level similar to the AP to BL direction.

**Cellular Accumulation of Pyrethroids**

To establish whether DLM, CPM, and TPM are substrates for P-gp-mediated efflux, the caco-2 cellular accumulation of each pyrethroid was evaluated with and without the P-gp inhibitors CSA and ritonavir. Surprisingly, CSA and ritonavir significantly reduced the accumulation of DLM, CPM, and TPM (Figs. 3A–C). Accumulation of all 3 pyrethroids was reduced approximately 3-fold to 4-fold in the presence of CSA and reduced approximately 1.5-fold to 2-fold with ritonavir. To establish whether DLM, CPM, or TPM can inhibit P-gp transport activity, the cellular accumulation of R6G with 10μM DLM, CPM, or TPM was evaluated (Fig. 3D). No change in cellular accumulation of R6G was observed in the presence of any of the pyrethroids. To further assess the potential P-gp transport properties of DLM, CPM, and TPM, the P-gp overexpressing cell line MDCK-MDR1 was utilized. The accumulation of R6G was significantly reduced in MDCK-MDR1 cells compared with wild-type MDCK, confirming functional overexpression of P-gp (Fig. 4A). In addition, CSA significantly increased accumulation of R6G in both MDCK and MDCK-MDR1 cells (Fig. 4A). No change in accumulation of DLM and CPM was observed between MDCK-MDR1 and MDCK cells (Figs. 4B and C). However, a slight, statistically significant decrease was found for TPM (Fig. 4D). Similar to caco-2 cells, the P-gp inhibitor CSA significantly decreased the uptake of DLM, CPM, and TPM in both MDCK-MDR1 and MDCK cells.

**Directional Flux of DLM, CPM, and TPM Across Caco-2 Monolayers**

For DLM and CPM, the AP to BL (absorptive) flux was greater than in the BL to AP (secretory) direction (Figs. 5A and B). The resulting $P_{app}$ values were significantly greater in the AP to BL than in the BL to AP direction for DLM and CPM (Table 1). Although trending to a greater AP to BL flux, the $P_{app}$ value was not statistically significant compared with the BL to AP $P_{app}$ value for TPM (Fig. 5C, Table 1). The ratio of the $P_{app}$ values (BL to AP/AP to BL) provides a means to assess the permeability process. Ratios > 1 suggest an efflux transport process, approximately 1 passive diffusion, and < 1 an absorptive influx transport. All 3 compounds had values less than 1, suggestive of an absorptive influx transport process (Table 1).

**FIG. 3.** Cellular uptake of 1μM DLM (A), 1μM CPM (B), and 1μM TPM (C) by caco-2 cells with or without the P-gp inhibitors CSA (25μM) or ritonavir (50μM) at 37°C for 3 min. Results are reported as the mean ± SE normalized to total protein with $N = 3$ independent experiments. (*) Statistically significant ($p < .05$) differences between pyrethroid alone and either CSA or ritonavir. D, Inhibitor properties for DLM, CPM, and TPM were determined by quantifying the accumulation of 0.5μM R6G in the presence of 10μM of each pyrethroid at 37°C for 3 min. Results represent the mean relative fluorescence (RFU) ± SE normalized to total protein with $N = 3$ independent experiments. Abbreviations: CPM, *cis*-permethrin; CSA, cyclosporine A; DLM, deltamethrin; TPM, *trans*-permethrin.
Because the reduced cellular accumulation of DLM, CPM, and TPM with CSA suggests inhibition of an absorptive influx transport process, effects of CSA on $P_{app}$ values for the AP to BL and the BL to AP directions were determined. When CSA was placed on the AP side, the $P_{app}$ in the AP to BL direction was significantly reduced for DLM and CPM (Figs. 6A and B). Although trending lower, the inclusion of CSA did not result in a significantly lower $P_{app}$ value for the AP to BL direction for TPM (Fig. 6C). No effects on the BL to AP $P_{app}$ values for any of the pyrethroids were found when CSA was placed on the AP side of the cell monolayers.

In order to compare the absorption potential of DLM, CPM, and TPM relative to established agents, the $P_{app}$ values of pyrethroids were contrasted with those of the low-permeability/low-absorption marker mannitol and the high-permeability/high-absorption drug propranolol. The AP to BL $P_{app}$ values for DLM, CPM, and TPM were substantially lower than those for propranolol (Fig. 7). The CPM $P_{app}$ value resembled that of the low-permeability marker mannitol, although DLM and TPM exhibited modestly higher values.

**Transport Kinetics of DLM, CPM, and TPM**

To evaluate DLM, CPM, and TPM transport kinetics in caco-2 cells, concentration-dependent uptake was assessed. Because previous experiments have established that CSA at 25μM maximally inhibited DLM, CPM, and TPM uptake (data not shown), the inclusion of CSA in the transport assay allows for estimation of the nonsaturable uptake component. Figure 8 demonstrates that in the presence of CSA, transport of DLM, CPM, and TPM demonstrated linearity over the concentration range evaluated. To estimate the saturable transport kinetics of DLM, CPM, and TPM, equation 2 was fitted to the total and nonsaturable uptake components. A saturable curve (dotted line) was obtained for all 3 pyrethroids with $K_m$ and $V_{max}$ values listed in Table 2.

**DISCUSSION**

A wide variety of drugs and environmental contaminants have been shown to interact with P-gp (Abu-Qare et al., 2003). Some of these compounds are P-gp inhibitors, whereas others are substrates and are effluxed by cells expressing P-gp. Substrates for P-gp efflux typically possess a degree of hydrophobicity that allows for partitioning into the membrane lipid bilayer and subsequent binding to P-gp (Seelig and Landwojtowicz, 2000). Thus, the high degree of hydrophobicity of pyrethroids may suit them for P-gp transport activity. Experiments in the current study with caco-2 cells that exhibit functional P-gp transport, however, revealed no evidence of pyrethroid efflux. The absence of any substantial decrease in pyrethroid uptake by
PYRETHROID TRANSPORT IN CACO-2 CELLS

the P-gp overexpressing MDCK-MDR1 cells compared with wild-type further supports the lack of P-gp efflux transport. Moreover, DLM, CPM, and TPM did not function as inhibitors of P-gp activity, as demonstrated by no change in R6G accumulation in their presence. To our knowledge, no one has determined whether pyrethroids are P-gp substrates in enterocytes or other cells. Very high concentrations (250μM) of PM and fluvalinate modestly inhibited efflux of doxorubicin in murine melanoma cells transfected with the human MDR1 gene (Bain and LeBlanc, 1996). Cypermethrin and fenvalerate exhibited high affinity for P-gp and stimulated ATPase activity in reconstituted P-gp proteoliposomes, but no difference in toxicity of the pesticides was found in vitro between wild-type and P-gp-overexpressing cell lines (Sreeramulu et al., 2007). Fenvalerate was also found to be a weak P-gp inhibitor for calcein-AM efflux in P-gp-overexpressing mouse fibroblasts (Pivcevic and Zaja, 2006). P-gp inhibitors typically have a cyclic structure, molecular weights of 400–500, and LogP values from 3.6 to 4.5 (Bain and LeBlanc, 1996). Compounds with a LogP above 4.5 are apparently so highly partitioned into membrane lipids that interaction with, and quite possibly transport by P-gp is significantly limited (Bain and LeBlanc, 1997; Bain et al., 1997). The very high LogP values of DLM and PM (6.1 and 6.5, respectively) may have limited their interactions with and transport by P-gp in our intestinal absorption model.

Although unexpected, the accumulation of DLM, CPM, and TPM was significantly reduced in MDCK, MDCK-MDR1, and caco-2 cells by the well-established P-gp inhibitors CSA and ritonavir (Drewe et al., 1999; Tang et al., 2002). Nevertheless, the promiscuity of CSA and ritonavir to interact with influx as well as efflux transporters is well documented. Thus, the significant decreases in DLM, CPM, and TPM accumulation in the presence of CSA and ritonavir suggest involvement of an influx transport process in caco-2 cells. The greater AP to BL flux and transport ratios less than 1 for DLM, CPM, and TPM are additional evidence that an influx transport process is involved in the intestinal absorption of the pyrethroids. The attenuation of DLM, CPM, and TPM AP to BL \( P_{\text{app}} \) with inclusion of CSA on the AP surface and lack of impact on the BL to AP \( P_{\text{app}} \) suggest that the potential transporter is localized on the luminal surface.

Kinetic analysis of uptake demonstrated a nonlinear curve for all 3 pyrethroids, further suggesting that a saturable process is involved in their absorption. Both CSA and ritonavir can interact with overlapping and distinct influx transporters belonging to the organic anion-transporting polypeptide (OATP) family and can limit cellular accumulation of substrates (Annaert et al., 2010; Letschert et al., 2006). The expression of OATPs is widely found

### Table 1: Apparent Permeability \( (P_{\text{app}}) \) and Transport Ratios

<table>
<thead>
<tr>
<th>Compound</th>
<th>( P_{\text{app}} ) AP to BL (cm/s)</th>
<th>( P_{\text{app}} ) BL to AP (cm/sec)</th>
<th>Transport ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deltamethrin</td>
<td>( 7.6 \times 10^{-6} \pm 1.3^* )</td>
<td>( 3.4 \times 10^{-6} \pm 0.6 )</td>
<td>0.44</td>
</tr>
<tr>
<td>cis-Permethrin</td>
<td>( 2.4 \times 10^{-6} \pm 0.5^* )</td>
<td>( 1.1 \times 10^{-6} \pm 0.3 )</td>
<td>0.45</td>
</tr>
<tr>
<td>trans-Permethrin</td>
<td>( 4.7 \times 10^{-6} \pm 0.9 )</td>
<td>( 3.2 \times 10^{-6} \pm 0.9 )</td>
<td>0.68</td>
</tr>
</tbody>
</table>

Abbreviations: AP, apical; BL, basolateral.

*Transport ratio = \( P_{\text{app}} \) BL to AP/\( P_{\text{app}} \) AP to BL.

*Statistically significant compared with the BL to AP \( P_{\text{app}} \).

FIG. 5. Transepithelial flux of DLM (A), CPM (B), and TPM (C) across caco-2 monolayers grown on Transwell inserts. (*) AP to BL flux reflected the amount of pyrethroid appearing in the BL chamber solution when 1μM DLM, CPM, or TPM was placed on the AP side. (◻) BL to AP flux was determined by placing 1μM DLM, CPM, or TPM on the BL side and monitoring the amount that entered the AP side. Data are expressed as the cumulative amount transported across a unit area of caco-2 monolayers and represented as the mean ± SE with \( N = 3 \). Abbreviations: AP, apical; BL, basolateral; CPM, cis-permethrin; DLM, deltamethrin; TPM, trans-permethrin.
in epithelial and endothelial barrier tissues such as the intestine, liver, kidney, and blood-brain barrier and is primarily localized on luminal/AP absorptive surfaces (Roth et al., 2012). OATP transporters can recognize a diverse range of chemical species including anionic, cationic, neutral, and lipophilic compounds such as arsenic, statins, steroids, and the microtoxins phalloidin and microcystin (Hagenbuch and Gui, 2008). Although member(s) of the OATP family are candidate influx transporter(s) for DLM, CPM, and TPM, numerous other influx transporters are found in intestinal enterocytes belonging to the Solute Carrier superfamily of transporters (Englund et al., 2006). CSA and ritonavir inhibit OATPs and pyrethroid uptake by caco-2 cells, but our results do not identify which transport family is involved in DLM, CPM, and TPM absorptive transport.

An advantage of the caco-2 model system is the well-established correlation between $P_{app}$ and in vivo GI absorption (Artursson et al., 2001). Generally, $P_{app}$ values $<2 \times 10^{-6}$ cm/s predict low absorption (0%–20%) and values $>1 \times 10^{-5}$ cm/s predict high absorption (90%–100%; Press and Di Grandi, 2008). In the absence of in vivo absorption data for pyrethroids, the oral absorption potential was predicted based upon the $P_{app}$ values of the low-permeability/low-fraction-absorbed compound mannitol and the high-permeability/high-fraction-absorbed drug propranolol. Both compounds are used extensively as the upper and lower parameters for permeability correlations with in vivo fraction absorbed after oral administration (Yee, 1997). Although the absolute $P_{app}$ values from caco-2 cells have been well documented to vary between laboratories, correlations to the fraction absorbed still hold regardless of the quantitative value of the $P_{app}$ (Artursson et al., 2001). Therefore, $P_{app}$ values of mannitol (1.95 $\times 10^{-6}$ cm/s) and propranolol (2.5 $\times 10^{-5}$ cm/s) were experimentally determined to calibrate our caco-2 model system and were found to be consistent with the range of $P_{app}$ values designated for these low-absorption and high-absorption compounds, respectively (Press and Di Grandi, 2008). In comparison, the $P_{app}$ for CPM was similar to the low-permeability marker mannitol, but values for both TPM and DLM were somewhat higher. Nevertheless, $P_{app}$s of DLM, CPM, and TPM were substantially lower than for the high-permeability and well-absorbed compound propranolol, indicating they have low to moderate oral absorption potential in vivo. Although our

![FIG. 6. Permeability coefficients of DLM (A), CPM (B), and TPM (C) calculated from transepithelial flux measurements using equation 1. $P_{app}$ values for pyrethroids were determined with the P-gp inhibitor CSA (25 μM) only added to the AP chamber. Results are expressed as the mean ± SE with $N = 3$. (*) Statistically significant ($p < .05$) differences between AP and BL $P_{app}$ for pyrethroid alone to the AP and BL $P_{app}$ with CSA. Abbreviations: AP, apical; BL, basolateral; CPM, cis-permethrin; CSA, cyclosporine A; DLM, deltamethrin; $P_{app}$, permeability coefficient; TPM, trans-permethrin.](image)

![FIG. 7. Comparison of the AP to BL $P_{app}$ values of DLM, CPM, and TPM with the low permeability marker mannitol and high permeability marker propranolol. Results are expressed as the mean ± SE with $N = 3$. (*) Statistically significant ($p < .05$) differences compared with mannitol. Abbreviations: AP, apical; BL, basolateral; CPM, cis-permethrin; DLM, deltamethrin; $P_{app}$, permeability coefficient; TPM, trans-permethrin.](image)
findings demonstrate an apparent influx transport process contributing to the GI absorption of DLM, CPM, and TPM, comparison of the $P_{\text{app}}$ values suggests this process does not result in high absorptive flux but is of low capacity.

The high LogP values of DLM, CPM, and TPM would be indicative of high permeability and high oral absorption, based on the partitioning hypothesis for drug absorption. However, single-variable correlations such as LogP do not adequately predict the permeability of highly lipophilic compounds (Artursson et al., 2001). The caco-2 permeability of a series of lipophilic compounds reached a plateau at LogP values between 2 and 3.5, but permeability diminished significantly when LogP exceeded 4.0 (Wils et al., 1994). Reduced permeability at high LogP values may be due to poor solubility and/or membrane retention. Lipinski et al. (2001) published what they termed the “Rule of 5,” which predicts that compounds will have poor membrane penetration when they have > 5 H bonds; > 10 H bond acceptors; molecular weight > 500; and LogP > 5. These properties apply to many pyrethroids including DLM, CPM, and TPM, and likely also contributed to limiting the permeability in our caco-2 intestinal model.

Although Caco-2 cell monolayers and similar in vitro systems are quite useful in assessing GI permeability, they have limitations. Solubility of DLM, CPM, and TPM in our transport assay buffer was limited, even when low concentrations of glycerol formal (1%) were incorporated. Similar to previous reports, inclusion of HSA on the receiver side improved the permeability of the lipid-soluble chemicals by mimicking in vivo vascular “sink” conditions (Krishna et al., 2001). Other differences from in vivo conditions include the absence of CNS control on gut motility and the likelihood of dual absorption pathways for pyrethroids. Highly lipophilic compounds can be absorbed both into mesenteric venous blood and lymphatics. Jandacek et al. (2009) found that 83% of an oral dose of pentachlorophenol (LogP = 3.5) entered the portal blood of rats versus 11% of hexachlorobene (LogP = 5.7) and only 4.6% of DDT (LogP = 6.9). According to Charman and Stella (1986), LogP ≥ 5 is required for absorption via chylomicrons into the lymphatics. Consumption of such chemicals in digestible oil enhances lymphatic flow and uptake. In addition, caco-2 cells only provide a correlation for GI absorption and do not adequately describe overall bioavailability due to limited expression of CYP450 enzymes (Crespi et al., 1996). Thus, systemic exposure of DLM, CPM, and TPM may be further reduced by the action of CYP450 enzymes found in enterocytes, as well as first pass through the liver.

In conclusion, our results suggest that the efflux transporter P-gp does not appreciably restrict intestinal absorption of DLM or either isomer of PM. All 3 pyrethroids exhibited transport properties indicative of their being substrates for an influx transport process. Further work is required to establish

![FIG. 8. Kinetics of DLM (A), CPM (B), and TPM (C) uptake by caco-2 cells. Total cellular accumulation (*) of increasing concentrations of pyrethroids was assessed at 37°C for 3 min. The nonsaturable (diffusion) component (◻) of uptake was determined by the cellular accumulation of increasing concentrations of pyrethroids in the presence of CSA (25μM). The saturable pyrethroid uptake (dashed line) was estimated by curve fitting the total and nonsaturable uptake components. Data represents the mean ± SE with N = 3. Abbreviations: CPM, cis-permethrin; CSA, cyclosporine A; DLM, deltamethrin; TPM, trans-permethrin.]

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_m$ (μM)</th>
<th>$V_{\text{max}}$ (pmol/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deltamethrin</td>
<td>1.5±0.8</td>
<td>62.7±11.7</td>
</tr>
<tr>
<td>cis-Permethrin</td>
<td>2.8±0.7</td>
<td>16.9±1.85</td>
</tr>
<tr>
<td>trans-Permethrin</td>
<td>2.9±1.6</td>
<td>28.6±6.69</td>
</tr>
</tbody>
</table>
the family of transporters involved in pyrethroid uptake. Our in vitro findings on the apparent permeability coefficient values of DLM, CPM, and TPM are consistent with compounds having limited GI absorption in vivo. Although nonspecific and specific transport processes contribute to intestinal absorption, the low permeability of DLM, CPM, and TPM suggest limited oral absorption potential.

**FUNDING**

Council for the Advancement of Pyrethroid Human Risk Assessment.

**ACKNOWLEDGMENTS**

The authors would like to thank the Council for the Advancement of Pyrethroid Human Risk Assessment for funding of this work.

**REFERENCES**


