Carrier Mechanisms Involved in the Transepithelial Transport of bis(POM)-PMEA and Its Metabolites Across Caco-2 Monolayers

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Purpose. To investigate the role of carrier mechanisms in: [1] the polarized transport of the bis(pivaloyloxymethyl)- [bis(POM)-] ester prodrug of the antiviral agent 9-(2-phosphonylmethoxyethyl)adenine [PMEA] and [2] the directional secretion of its metabolites.

Methods. Caco-2 monolayers were used to study the modulating effect of carriers on the transport of bis(POM)-PMEA and the efflux of intracellularly formed metabolites mono(POM)-PMEA and PMEA from the cells. The interaction of bis(POM)-PMEA and its metabolites with the efflux mechanisms present in Caco-2 monolayers was investigated by testing the effect of various concentrations of verapamil (30, 100, 300 μM) or indomethacin (10–500 μM) on transport and efflux.

Results. Polarity in transport of bis(POM)-PMEA (50 μM) across Caco-2 monolayers was noted: transport of total PMEA [=bis(POM)-PMEA, mono(POM)-PMEA and PMEA] was significantly higher in basolateral (BL) to apical (AP) direction (14.5 ± 0.4%) than transport in the opposite (AP to BL) direction (1.7 ± 0.2%). This difference was reduced in a concentration dependent way when verapamil (0–100 μM) was included in both AP and BL incubation media. After loading the cells with bis(POM)-PMEA (100 μM) for 1 hr, studies on efflux of PMEA and mono(POM)-PMEA from the Caco-2 monolayers over a 3 hr period, revealed that both metabolites were preferentially secreted towards the AP compartment. Efflux of PMEA towards AP and BL compartments amounted to 14.6 ± 1.1% and 5.3 ± 0.4%, respectively, of the initial intracellular amount of total PMEA, while efflux of mono(POM)-PMEA towards AP and BL compartments was limited to 2.3 ± 0.1% and 0.5 ± 0.1%, respectively. When 10 μM indomethacin was included in the AP incubation medium, efflux of PMEA was decreased to 7.8 ± 0.3% and 3.3 ± 0.3% towards the AP and BL compartments, respectively. The decrease in efflux by indomethacin was concentration-dependent up to 100 μM. Transepithelial transport of total PMEA was also reduced in the presence of 30 μM indomethacin, as reflected in smaller concentrations of PMEA and mono(POM)-PMEA in the acceptor compartment, irrespective of the transport direction.

Conclusions. The data obtained in this study suggest that bis(POM)-PMEA is substrate for a P-glycoprotein-like carrier mechanism in Caco-2 monolayers, while its metabolites mono(POM)-PMEA and PMEA are transported by a non-P-glycoprotein efflux protein.

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INTRODUCTION

The antiviral agent 9-(2-phosphonylmethoxyethyl)adenine (PMEA, adefovir) is a lead compound of the class of acyclic nucleoside phosphonate (ANP) analogues and displays broad-spectrum antiviral activity. PMEA has been shown to be a strong inhibitor of the replication of retro-viruses (human immunodeficiency virus type 1 and 2, HIV-1 and -2), herpes viruses and hepadna viruses (hepatitis B virus, HBV) (1). The bis(pivaloyloxymethyl)-ester of PMEA (adefovir dipivoxil) has been developed as an ester prodrug of the compound to bypass the low oral bioavailability of PMEA (< 1% in monkeys) and is currently undergoing Phase II/III clinical trials in HIV-1- and HBV-infected patients. Studies with bis(POM)-PMEA administered in single doses of 125 to 500 mg in men showed that the oral bioavailability of PMEA was approximately 35% (2).

We previously confirmed the increase in transport across Caco-2 monolayers [a generally accepted *in vitro* model for drug transport studies (3,4,5,6)] of total PMEA [i.e. bis(POM)-PMEA, mono(POM)-PMEA and PMEA] after apical administration of bis(POM)-PMEA (7). In an additional study (8), the intestinal metabolism and transport of several S-acyl-thioethyl [SATE] esters of PMEA were investigated and compared to bis(POM)-PMEA using the Caco-2 model as a selection tool for compounds with potential oral absorption.

The present study was undertaken in order to investigate the previously observed polarity in transport of bis(POM)-PMEA and the polarized secretion of its metabolites (7) as well as to further explore the relevance of the Caco-2 test system to investigate intestinal transport mechanisms of ester prodrugs. Carrier efflux systems have been extensively described as mechanisms modulating drug transport, and their presence in Caco-2 monolayers has already been documented in literature. The presence of a P-glycoprotein (P-gp) related efflux mechanism in the apical membrane of Caco-2 cells (9) and other epithelial or endothelial cell lines (CV-1 (10), BBMEC (11)) is frequently invoked to explain polarity in transport of a wide range of structurally diverse lipophilic and/or cationic compounds. Vinblastine (12), chlorpromazine (13), celiprolol (14), digoxin (15), cyclosporin A (16) and several peptides (17) have all been reported to be substrates for this efflux mechanism. Although expression of this transporter is higher in Caco-2 monolayers as compared to normal enterocytes, the functional presence of this protein has been demonstrated in normal epithelia of gut, kidney and blood brain barrier, where it is believed to play a significant biochemical barrier function (18). Besides the presence of this P-gp related apically located efflux mechanism, Allen *et al.* (19) have shown evidence for the presence of a second carrier mechanism responsible for the efflux of anionic compounds. They demonstrated that the negatively charged fluorochrome 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein is a substrate for this carrier system, which is distinct from the mdr1 gene product P-gp and is present in various epithelial cell lines (HCT-8, T84, HGT-1 and MDCK). Additional experiments showed that secretion of this compound in MDCK- and Caco-2 monolayers was polarized towards the apical compart-

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ment, and that this efflux system was inhibited by indomethacin and 5-nitro-2-(3-phenylpropyl-amino)-benzoate (20).

In this study, the possible involvement of these carrier mechanisms in polarized transport and directional secretion of bis(POM)-PMEA and its metabolites was investigated in Caco-2 monolayers by monitoring the effects of specific inhibitors, i.e. verapamil and indomethacin, that block P-gp-related and non-P-gp-related efflux mechanisms, respectively.

MATERIALS AND METHODS

Materials

PMEA and bis(POM)-PMEA were obtained from Dr. N. Bischofberger (Gilead Sciences, Foster City, CA). Tetrabutylammonium hydrogen sulfate (Fluka, Switzerland), potassium dihydrogen phosphate (Merck, Darmstadt, Germany), methanol and acetonitrile (BDH, Poole, UK) were HPLC grade. All chemicals used for culturing the Caco-2 cells were purchased from Gibco Inc. (Life Technologies, Belgium). D-(+)-glucose and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, Mo). Sodium fluorescein, sodium hydroxide and ammonia 25% (v/v) solution were obtained from UCB (Leuven, Belgium). DMSO was obtained from Acros (Geel, Belgium). Transport medium (TM) consisted of 500 ml Hanks' Balanced Salt Solution (HBSS) containing 25 mM glucose and 10 mM Hepes. Cell culture medium consisted of DMEM supplemented with 10% Fetal Bovine Serum, 1% MEM-non-essential amino acids solution and 100 IU/ml penicillin-streptomycin.

Caco-2 Cell Culture

Caco-2 cells were kindly provided by Dr. Y. Schneider (UCL, Belgium). Cells were grown in 75 cm^2 culture flasks at 37°C in an atmosphere of 5% CO_2 and 90% relative humidity. Cells were passaged every 3–4 days (at 70–80% confluence) at a split ratio of 1 to 7. Cells were negative for Mycoplasma infection. Validation of the use of the Caco-2 model system in our laboratory was described previously (21).

Growth of Cells on Membrane Inserts and in Wells

For transport experiments, Caco-2 cells were plated at a density of 40,000 cells/cm^2 on Anopore membrane inserts (0.2 μm pore diameter, 25 mm diameter; Nunc, Roskilde, Denmark). Confluence was reached within 3–4 days after seeding and the monolayers were used for the experiments between days 18–24 post-seeding. Cell passages between 114 and 127 were used in the experiments.

Test Solutions

Solutions of bis(POM)-PMEA in TM were prepared by spiking TM with a concentrated stock solution (50 mM) made up in DMSO. The final DMSO concentration in solutions used for experiments with the monolayers was always adjusted to 0.2% DMSO. Preliminary experiments had shown that concentrations up to 2% DMSO did not affect cell monolayer integrity during a 3 hr incubation period (as could be concluded from stable TEER values throughout the experiments).

HPLC-Analysis of PMEA Prodrugs and Their Metabolites

Bis(POM)-PMEA and its metabolites were analyzed using a high-performance liquid chromatographic system equipped with a Model 600 Controller and Pump, a Model 717 plus autosampler and a Model 480 Lambda-Max UV detector at 260 nm (Waters, Milford, MA). UV-signals were monitored and the obtained peaks integrated using a personal computer running Waters Maxima 820 chromatography software. The column used was a Waters SymmetryShield C8 (4.6 × 150 mm). Detailed chromatographic conditions were described previously (7).

In order to prevent chemical degradation of the esters at the end of the incubation period, the pH of the samples (containing solutions of TM at pH 7.4) was adjusted to 3.3 by adding HCl 0.05 N. In addition, the samples were kept at 4°C in order to minimize chemical degradation of the bis-esters during storage in the auto-injector. Concentrations of PMEA and the bis-ester were determined using calibration graphs made up by standards of each compound. Concentrations of mono-esters were calculated using bis-ester calibration curves.

Transport Experiments

Transport of the ester prodrugs across Caco-2 monolayers was studied using monolayers 18–24 days post-seeding. Before the experiments, the monolayers were rinsed twice with TM and preincubated for 30 min. After the preincubation, transepithelial electrical resistance (TEER) of the monolayers was measured at 37°C using a Millicel ERS apparatus (Millipore) to check cell monolayer integrity. Only monolayers displaying TEER values above 400 $\Omega \cdot cm^2$ were used in the experiments. Transport was initiated by adding 2 ml of TM to the acceptor side and 2 ml of a solution of test compound (50 μM) to the donor side. When the effect of verapamil was tested it was included in either the AP compartment during incubation or in both compartments during incubation and preincubation as stated in legends to Figures and Tables. When the effect of indomethacin was tested it was added to the apical side during incubation. Preliminary studies had shown that adding indomethacin to only one side or to both sides of the monolayers did not result in different effects on transport or efflux of bis(POM)-PMEA and its metabolites. At predetermined time points (60, 120, 180 min), samples (100 μl) were taken from the acceptor side and replaced by 100 μl of fresh TM in order to maintain the same volume. The dilution was taken into account during further calculations. At the end of the experiments (180 min), samples were also taken from the donor compartment. All monolayers were checked for integrity after the experiment by measuring TEER values as well as transport of the paracellular leakage marker sodium fluorescein (by measuring the UV-absorption of the basolateral solution at 490 nm, 1 hr after adding a solution of 1 mg/ml sodium fluorescein to the apical side).

Efflux of PMEA and Mono(POM)-PMEA from Caco-2 Monolayers

Caco-2 monolayers (grown on inserts) were incubated with 100 μM bis(POM)-PMEA at the apical side for 1 hr ("loading"). The monolayers were washed twice with ice-cold TM and incubated with fresh TM (37°C). The last wash solution was

analyzed for the absence of bis(POM)-PMEA. The efflux of PMEA and mono(POM)-PMEA by the cells was measured as a function of time by analyzing apical and basolateral incubation media. When the effect of indomethacin or verapamil was tested, they were included in the apical incubation medium or in both incubation media, respectively. The total intracellular amount after loading was determined according to a previously described procedure (7). Briefly, cells were rapidly washed two times with ice-cold TM to arrest uptake and/or efflux, whereafter the monolayers were homogenized with 2 ml of methanol:water (70:30) at −20°C to denaturate the proteins. After centrifugation at 3000 g for 5 min, the supernatant was injected for determination of the intracellular metabolites. Efflux was expressed as percentage of the total intracellular amount immediately after loading.

Degradation of the PMEA-Esters by Cell Homogenates

Freshly scraped Caco-2 monolayers grown in a 75 cm^2 flask were homogenized in 10 ml TM using a cell disrupter (Branson Sonifier B 15) for 10 sec at 4°C. After centrifugation at 13,000 g for 5 min, the supernatant was harvested and kept at 4°C. The degradation profile of bis(POM)-PMEA was assessed at 37°C by spiking Caco-2 homogenate with bis-(POM)-PMEA to obtain a final concentration of 10 μM in the presence or absence of 100 μM verapamil or indomethacin. At predetermined time points, 100 μl samples were taken from the mixture and added to an equal volume of ice-cold methanol. After vortexing and centrifugation at 13,000 g for 5 min, the supernatant was analyzed according to the HPLC method described.

RESULTS AND DISCUSSION

In the present study, the modulation of transport of bis-(POM)-PMEA and polarized secretion of its metabolites in Caco-2 monolayers was investigated by studying transport and efflux in the presence of specific inhibitors of carrier mechanisms. In a first part of this study, the influence of the P-glycoprotein (P-gp) related efflux carrier on transport of total PMEA [=bis (POM)-PMEA and its metabolites mono(POM)-PMEA and PMEA] was investigated after the addition of bis-(POM)-PMEA to Caco-2 monolayers. Previous experiments in our laboratory had shown polarity in transport of total PMEA across Caco-2 monolayers (7): when bis(POM)-PMEA was added to the apical side of Caco-2 monolayers, transport of total PMEA was significantly lower as compared to transport in the opposite direction.

Figure 1 illustrates the more than 8-fold higher transport of total PMEA in the secretory direction then in the absorptive direction after adding bis (POM)-PMEA (50 μM) to the monolayers. This effect was concentration-dependent since the ratio of 'basolateral to apical' (BL → AP) over 'apical to basolateral' (AP → BL) transport for initial bis(POM)-PMEA concentrations of 100 μM and 10 μM was 2.2 and 13.0, respectively. Concentration-dependent metabolism of bis(POM)-PMEA in Caco-2 monolayers, which was suggested previously (7) to explain concentration-dependent transport, could not be invoked to explain this observation. Therefore, the involvement of a P-gp related drug efflux mechanism located in the apical membrane of Caco-2 monolayers and responsible for efflux of

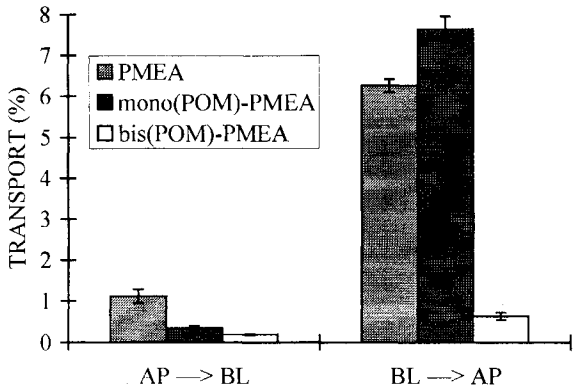

Fig. 1. Transport of bis(POM)-PMEA (50 μM) across Caco-2 monolayers in the apical to basolateral (AP → BL) and opposite (BL → AP) direction as illustrated by the appearance of bis(POM)-PMEA, mono(POM)-PMEA and PMEA in the acceptor compartment. Bars represent cumulative amounts of each metabolite after 180 min and transport is expressed as percentage (±SD) of the amount of bis(POM)-PMEA initially added to the donor compartment (n = 3).

a wide variety of cationic and/or lipophilic molecules, was taken into account to explain the observed polarity in transport. To test this hypothesis, the effect of various concentrations of the commonly used P-gp inhibitor and Ca^{++}-influx antagonist verapamil on transport of total PMEA was investigated for both transport directions. Data on the effect of verapamil on transport of total PMEA are shown in Figure 2: there was an increased transport of total PMEA in the absorptive direction and reduced transport in the secretory direction when verapamil concentrations were increased from 0 to 100 μM. Degradation of bis-(POM)-PMEA in Caco-2 homogenate was not affected by the presence of verapamil (data not shown), indicating that verapamil did not interfere with bis(POM)-PMEA metabolism. The data presented in Table 1 illustrate that the higher absorptive (AP → BL) transport of total PMEA in the presence of verapamil is reflected in higher basolateral concentrations of bis(POM)-

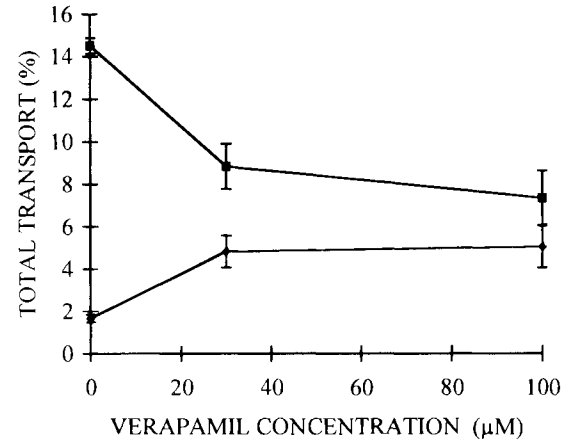

Fig. 2. Effect of the various concentrations of verapamil (added to both sides of the monolayers) on transport of total PMEA in either apical to basolateral (◆) or the basolateral to apical (■) direction after addition of 50 μM bis(POM)-PMEA to the donor compartment. Values shown represent total cumulative amounts after 180 min. Transport is expressed as average percentage (±SD) of the amount of bis(POM)-PMEA initially added to the donor compartment (n = 3).

Table 1. Effect of Verapamil on the Concentration of Bis(POM)-PMEA and Its Metabolites in the Acceptor Compartment of Caco-2 Monolayers 3 hr After Applying Bis(POM)-PMEA to the Cell Monolayers.

	AP → BL direction		BL → AP direction	
	control	30 μ*M* verapamil	control	30 μ*M* verapamil
PMEA	1.70 ± 0.04	2.39 ± 0.05	5.69 ± 0.26	4.94 ± 0.25
mono(POM)-PMEA	0.86 ± 0.05	1.36 ± 0.08	7.40 ± 0.21	5.86 ± 0.53
bis(POM)-PMEA	0.15 ± 0.01	0.45 ± 0.04	0.62 ± 0.05	0.33 ± 0.03

[a] Values shown represent percentages (± SD) of the initial amount of bis(POM)-PMEA added to the cells (n = 3).

PMEA (200% increase) as well as increased concentrations of both mono(POM)-PMEA (58% increase) and PMEA (41% increase). This could be attributed to increased intracellular concentrations of PMEA and mono(POM)- PMEA as the consequence of diminished activity of the efflux-system in the presence of the efflux inhibitor verapamil. The higher basolateral concentrations of mono(POM)-PMEA may also be explained by enhanced basolateral appearance of bis(POM)-PMEA and subsequent chemical hydrolysis. In the secretory (BL → AP) direction, verapamil induced a net decrease in transport: a reduction of 47%, 21% and 13% in the apical concentrations was observed for bis(POM)-PMEA, mono(POM)-PMEA and PMEA, respectively (Table 1). Further increase of the verapamil concentration from 100 to 300 μM resulted in higher transport of total PMEA, irrespective of the transport direction. This can be explained by the fact that at verapamil concentrations above 100 μM, membrane integrity was compromised (as could be concluded from reduced transepithelial electrical resistance (TEER) values and increased sodium fluorescein transport at the end of the incubation, probably resulting in significant contribution of the paracellular transport pathway to total transport).

In the next part of our study, the possible involvement of a non-P-gp efflux carrier mechanism in polarized secretion of mono(POM)-PMEA and PMEA from Caco-2 monolayers was investigated. Previous experiments in our laboratory had shown polarized efflux of the negatively charged metabolites PMEA and mono(POM)-PMEA from Caco-2 monolayers (7): after loading the cells with 100 μM bis(POM)-PMEA for 1 hr, efflux of both metabolites was higher towards the apical compartment as compared to efflux towards the basolateral compartment. Indomethacin has been described previously as an inhibitor of a non-P-gp efflux protein (in Caco-2 monolayers and other cell lines) which preferentially transports negatively charged compounds (20). To investigate whether mono(POM)-PMEA and PMEA could be substrates for this carrier system, efflux of these negatively charged metabolites was studied in the presence of various concentrations of indomethacin. These experiments revealed a concentration-dependent reduction in efflux of PMEA and mono(POM)-PMEA towards both sides of the monolayers for indomethacin concentrations between 0 and 100 μM (Figure 3). In the presence of 10 μM indomethacin, efflux (in % of intracellular amount immediately after loading the cells) of PMEA decreased from 14.6 ± 1.1% to 7.8 ± 0.3% in the apical direction and from 5.3 ± 0.4% to 3.3 ± 0.3% in the basolateral direction. When the indomethacin concentration was further increased from 100 to 500 μM, the effect was partly reversed, which was probably caused by a toxic effect of indomethacin on the cell monolayers. The higher efflux of PMEA as compared to mono(POM)-PMEA is likely due to the much higher intracellular levels of PMEA which has been demonstrated during uptake studies with bis(POM)-PMEA (7). As efflux towards both directions is influenced by the presence of indomethacin, the presence of a non-P-gp efflux carrier mechanism in both the apical and basolateral membrane of Caco-2 monolayers is suggested. The fact that verapamil (at concentrations that significantly alter total PMEA transport) did not significantly affect efflux of PMEA and mono(POM)-PMEA (data not shown) further suggests that both a P-gp and a non-P-gp efflux carrier mechanism are involved in the transport of bis(POM)-PMEA and the efflux of its metabolites.

To determine whether this non-P-gp carrier mechanism significantly contributed to modulation of transepithelial transport of total PMEA, total transport after administration of bis-(POM)-PMEA was determined in the absence or presence of indomethacin or a combination of indomethacin and verapamil. As shown in Figure 4, significantly lower amounts of PMEA and mono(POM)-PMEA were obtained in the basolateral compartment in the presence of 30 μM indomethacin in the apical incubation medium. Similar results were obtained when the transport was studied in the opposite direction (data not shown). Irrespective of the transport direction, the amounts of bis(POM)-PMEA in the acceptor compartments were not significantly altered in the presence of indomethacin. These results corroborate the conclusion that PMEA and mono(POM)-PMEA, but not bis(POM)-PMEA, are substrates for a non-P-gp efflux carrier

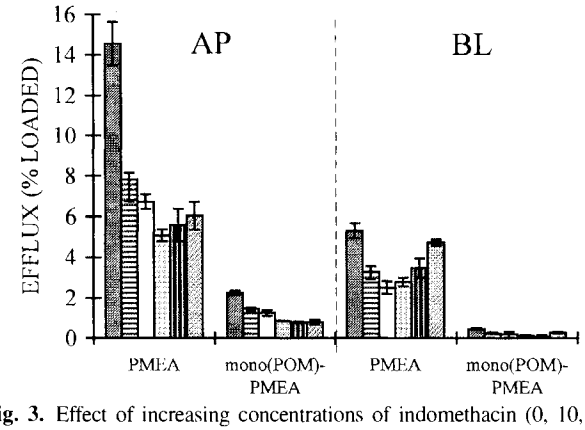

Fig. 3. Effect of increasing concentrations of indomethacin (0, 10, 30, 100, 300, 500 μM, from left to right) on efflux of PMEA and mono(POM)-PMEA towards the apical (AP) and basolateral (BL) side of Caco-2 monolayers, after loading the cells with 100 μM bis(POM)-PMEA for 60 min. Bars represent total cumulative amounts after 180 min. Efflux is expressed as average percentage (±SD) of the amount of total PMEA present inside the cells immediately after loading (n = 3).

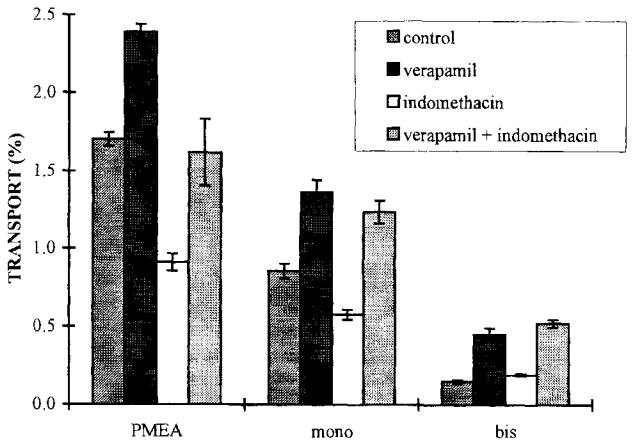

Fig. 4. Efffect of verapamil (30 μM apical), indomethacin (30 μM apical) and the combination of both compounds (30 μM each apical) on the concentrations of PMEA, mono(POM)-PMEA and bis(POM)-PMEA in the basolateral (acceptor) compartment of Caco-2 monolayers 180 min after adding 50 μM bis(POM)-PMEA to the apical side. Transport is expressed as cumulative percentage (±SD) of the amount of bis(POM)-PMEA initially added to the monolayers (n = 3).

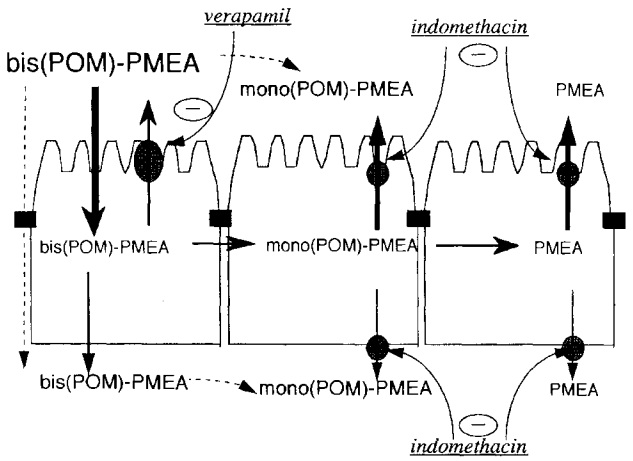

Fig. 5. Hypothetical scheme illustrating intestinal transport and metabolism of bis(POM)-PMEA and the involvement of distinct carrier mechanisms. Following intracellular uptake, bis(POM)-PMEA can either be effluxed back towards the apical compartment by a P-gp related carrier mechanism or undergo ester hydrolysis with formation of mono(POM)-PMEA. Alternatively, depending on the initial concentration, a limited amount of the intact ester may reach the basolateral compartment. The paracellular pathway could also, albeit to a limited extent, contribute to basolateral bis(POM)-PMEA. Intracellularly formed mono(POM)-PMEA is either effluxed towards apical and basolateral compartments or further converted to PMEA, which on its turn is also substrate for the non-P-gp transporter.

protein present in both apical and basolateral membranes of Caco-2 monolayers. Figure 4 also shows the combined effect of verapamil and indomethacin on transepithelial transport of total PMEA after 3 hr. This provides additional evidence for distinct carrier mechanisms responsible for the efflux of bis-(POM)-PMEA and the efflux of its metabolites mono-(POM)-PMEA and PMEA. Metabolism of bis(POM)-PMEA was not influenced by the presence of indomethacin at concentrations below 100 μM as could be concluded from degradation profiles of bis(POM)-PMEA in Caco-2 homogenate in the absence or presence of indomethacin (data not shown).

In conclusion, it is hypothesized that at least two distinct carrier mechanisms are involved in the polarized transport of bis(POM)-PMEA and the directional secretion of its metabolites. An hypothetical model illustrating the intestinal metabolism and modulation of bis(POM)-PMEA transport and efflux of its metabolites by these carrier mechanisms in Caco-2 monolayers is shown in Figure 5. Data obtained in this study illustrate the usefulness of the Caco-2 system as a comprehensive test tool to study intestinal transport and metabolism of ester prodrugs: the simultaneous effect of passive diffusion, metabolic degradation, chemical hydrolysis and distinct carrier mechanisms can be studied. This is an important advantage as compared to other absorption models (octanol/water partition coefficient, cell homogenates, . . .) which allow the investigation of only one process at a time. Additional experiments (*ex vivo, in situ* intestinal perfusion, *in vivo*) are required to further establish the relative contribution and quantitative significance of all the factors involved in the oral delivery of bis(POM)-PMEA *in vivo*.

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