Comparison of the Disposition of Ester Prodrugs of the Antiviral Agent 9-(2-phosphonylmethoxyethyl)adenine [PMEA] in Caco-2 Monolayers

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Purpose. To evaluate the potential of several bis-ester prodrugs of the antiviral agent 9-(2-phosphonylmethoxyethyl)adenine (PMEA, adefovir) to enhance the oral absorption of PMEA.

Methods. Caco-2 monolayers were used to estimate intestinal transport and metabolism of the bis(pivaloyloxymethyl)-ester [bis(POM)-] and a series of bis(S-acyl-2-thioethyl)-esters [bis(SATE)-] of PMEA. An LC-MS method was used for the identification of unknown metabolites which were formed from the SATE-esters.

Results. During transport across Caco-2 monolayers, all esters were extensively degraded as could be concluded from the appearance of the mono-ester and free PMEA in apical as well as basolateral compartments. Incubation of SATE-esters with the monolayers resulted in the formation of two additional metabolites, which were identified as 2-thioethyl-PMEA and its dimerisation product. All ester prodrugs resulted in enhanced transepithelial transport of total PMEA (i.e. the bis-esters and their corresponding metabolites, including PMEA), but significant differences could be observed between the various esters. Transport of total PMEA ranged from 0.4 ± 0.1% for the bis[S(methyl)ATE]-ester to 15.3 ± 0.9% for the more lipophilic bis[S(phenyl)ATE]-PMEA. A relationship between total transport of the esters and their lipophilicity (as estimated by their octanol/water partition coefficient) was established (r^2 = 0.87). Incubation of prodrug esters with homogenates from Caco-2 cells showed large differences in susceptibility of the compounds to esterases, the half-lives of the bis-esters varying from 4.3 ± 0.3 min for the bis[S(phenyl)ATE]-PMEA to 41.5 ± 0.8 min for its methyl analogue. In addition, intracellularly formed PMEA was observed to be further converted by the cells to the diphosphorylated PMEA (PMEApp).

Conclusions. Several SATE-esters of PMEA can be considered as potential alternatives to bis(POM)-PMEA, due to enhanced epithelial transport, sufficient chemical and enzymatic stability and adequate release of PMEA. Toxicological studies as well as *in vivo* experiments are required in order to further explore the potential of those SATE-esters as prodrugs for oral delivery of PMEA.

KEY WORDS: prodrugs; Caco-2; intestinal permeability; intestinal metabolism; antiviral; drug transport; PMEA.

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INTRODUCTION

The antiviral agent 9-(2-phosphonylmethoxyethyl)adenine (PMEA, adefovir) is the prototype of the acyclic nucleoside phosphonate (ANP) analogues and displays broad-spectrum antiviral activity. Due to a selective interaction of its diphosphorylated anabolite PMEApp with the reverse transcriptase or viral DNA polymerase, 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 (herpes simplex virus type 1 and 2) and hepadna viruses (hepatitis B virus, HBV) (1). The presence of a phosphonate group in these compounds offers the advantage that the ANP analogues circumvent the first phosphorylation step to the nucleoside-5′-monophosphate, catalyzed by cellular kinases such as thymidine kinase (2). In addition, the ANP analogues are enzymatically very stable, due to resistance of the C-P linkage to catabolic enzymes. Unfortunately, the negative charge associated with this phosphonate group at physiological pH (pK_{a1} = 2.0 and pK_{a2} = 6.8; (3)) is responsible for the low permeation of PMEA across biological membranes, which results in limited oral bioavailability of PMEA (4,5,6). Therefore, lipophilic esters of PMEA were synthesized in order to mask the negative charges and to obtain membrane-permeable prodrugs (Figure 1). The bis(pivaloyloxymethyl)-ester of PMEA [bis(POM)-PMEA, adefovir dipivoxil] is one of these prodrugs which is currently undergoing Phase II/III clinical trials in HIV-1- and HBV-infected patients. The bis(pivaloyloxymethyl)-ester of PMEA was initially selected as a potential membrane-permeable prodrug of PMEA by Shaw and Cundy (7), because of its enhanced transport across Caco-2 monolayers, which are generally accepted as an *in vitro* model for drug transport studies (8,9,10,11).

We previously confirmed the increase in transport across Caco-2 monolayers of total PMEA [i.e. bis(POM)-PMEA, mono(POM)-PMEA and PMEA] after apical administration of bis(POM)-PMEA (12). Bioavailability of PMEA after oral administration of its bis(POM)-ester was reported to be 53%, 18% and ~25% in mice, rats and cynomolgus monkeys, respectively (1). Studies with bis(POM)-PMEA administered in single doses of 125 to 500 mg in men revealed an oral bioavailability for PMEA of approximately 35% (13). In an attempt to further improve the oral absorption of PMEA, Benzaria *et al.* (14) have synthesized a series of carboxylesterase-labile bis(S-acyl-2-thioethyl)-[bis(SATE)-] ester prodrugs of PMEA (Figure 1), displaying high chemical stability in aqueous solution (buffer pH 7.2) and enhanced enzymatic stability in human serum. The presence of a thioester bond in addition to the phosphonate ester allows removal of the promoiety through mediation by carboxylesterases; hence, bioactivation of the SATE-esters is ensured due to the ubiquitous presence of carboxylesterases. The use of SATE ester prodrugs has been shown to be a successful approach to improve the *in vitro* anti-viral activity of isoddA due to increased intracellular delivery (15).

Using Caco-2 monolayers as a simulation of the intestinal mucosa, we have now evaluated these SATE-esters of PMEA as compared to bis(POM)-PMEA for their transepithelial transport and metabolism characteristics.

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MATERIALS AND METHODS

Materials

PMEA was kindly provided by Dr. A. Holý (Czech Academy of Sciences, Prague, Czech Republic); bis(POM)-PMEA was obtained from Dr. N. Bischofberger (Gilead Sciences, Foster City, CA, USA). Bis-S-acyl-thioethyl-esters were synthesized as previously described (14). 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 Janssen Chimica (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.

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). For uptake experiments, the cells were seeded directly in 6-well plates at the same density. 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, bis[S(methyl)ATE]-PMEA, bis[S(isopropyl)ATE]-PMEA, bis[S(tert-butyl)ATE]-PMEA and bis[S(phenyl)ATE]-PMEA in TM were prepared by spiking TM with a concentrated stock solution (50 mM) made up in DMSO. Final DMSO concentrations in solutions used for incubation of the monolayers were always adjusted to 0.2% DMSO. Preliminary experiments had shown that concentrations up to 2% DMSO did not affect cell monolayer integrity.

HPLC-Analysis of PMEA Prodrugs and Their Metabolites

All esters used and their metabolites were analyzed using a high-performance liquid chromatographic system equipped with a Model 600 Controller and Pump, a Model 717plus 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 Symmetry C8 (4.6 × 150 mm). Bis(POM)-PMEA and its metabolites were analyzed as described previously (12). The gradient programs were adapted if necessary to allow analysis of the SATE-esters.

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 stored 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. The additional metabolites formed during degradation of the SATE-esters (M1 and M2) were determined using PMEA calibration curves. As the dimerisation product (M2) eventually leads to the formation of PMEA, concentrations of this metabolite were expressed as PMEA concentrations. Although this calculation implies an overestimation of the concentrations of the product by a factor two, it allowed to gain a better insight in the amount of "total PMEA".

Determination of PMEA and PMEApp Using Ion-Exchange Chromatography

Intracellular concentrations of PMEA and PMEApp were determined using a Partisphere Sax column (4.6 × 125 mm) as described previously (16). The flow rate was 2 ml/min and the injection volume 100 μl. The eluent was monitored with a UV detector at 260 nm. The method allowed to trace PMEA, PMEAp and PMEApp in the presence of AMP, ADP and ATP. Concentrations of PMEA and PMEApp were calculated based on calibration curves made up with standards containing known concentrations of the compounds (5–100 μM).

Identification of Additional SATE-Metabolites Using LC-MS

Besides the mono-ester and PMEA, additional metabolites were observed during transport and metabolism studies with the bis[SATE]-esters. Samples containing these metabolites were subjected to mass spectrometry coupled liquid chromatography (LC-MS) for identification of these metabolites. HPLC instrumentation included a Waters LC System [Model 717 plus autosampler, Model 616 pump with column heater (set at 30°C) and controller 600S, Model 996 photodiode array detector] coupled to a Millennium Chromatography Manager System. The mass spectrometer (SSQ 7000, Finnigan-Mat) was directly coupled to the outlet of the UV-detector, and the full flow (1 ml/min) was allowed to penetrate into the Electrospray source, which was configured either in the negative or positive modes (ESI^- or ESI^+). A molar solution S (triethylammonium acetate buffer, pH 6.6) was filtered (Millex GVWP, 0.22 μm, Millipore) and stored at 5°C. Eluents A (S 20 ml, water to 1000 ml) and B (S 10 ml, acetonitrile 250 ml, water to 500 ml) were prepared daily and degassed by sonication and continuous Helium sparging. Crude samples (80 μl) were injected into the

analytical column (Hypersil ODS, 100 × 4.6 mm, 3 μm particle size, Hypersil, Eragny, France) protected by a precolumn (Guard-Pak inserts Delta-Pak C18 100 Å, Waters, Saint-Quentin, France) in ion-pairing conditions (eluent A). Then, the eluting strength was increased (slow linear gradient in 20 min from A to 60%A – 40%B, followed by a fast linear gradient in 5 min to 100% B).

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 (100 μM) to the donor side. At predetermined time points (60, 120, 180 min), samples (100 μl) were taken from the basolateral 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 (apical) 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).

Uptake Experiments

Uptake of test compounds was determined using Caco-2 monolayers grown in 6-well plates. Before the experiments, the monolayers were rinsed twice with TM (37°C), preincubated for 30 min, followed by incubation with test compound. After an incubation time of 180 min, monolayers were washed twice with ice-cold TM to arrest uptake and/or efflux, immediately followed by the addition of three milliliters of a mixture of 70 % methanol in water (–20°C) in order to denature proteins and to arrest enzymatic as well as chemical hydrolysis. Preliminary experiments had shown that no further degradation of the esters occurred during sample processing. The suspension obtained was centrifuged for 5 min (3,000 g, –20°C) and 25 μl of supernatant was analyzed according to the procedure described. Uptake of the compounds and their corresponding metabolites was expressed as nmol per mg of protein. The protein content of the monolayers was determined according to the method of Lowry (17) using bovine serum albumin as a standard.

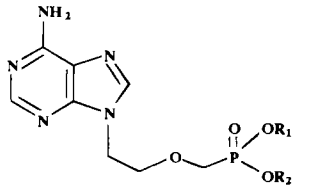
Degradation of the PMEA-esters by Cell Homogenates

Freshly scraped Caco-2 monolayers grown in a 75 cm^2 flask were homogenized in 10 ml TM using a cell disrupter (Branson Sonifier B15) 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 each ester was determined in triplicate at 37°C by spiking Caco-2 homogenate with bis-ester to obtain a final concentration of 10 μM. 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. Rate constants for the formation of the various metabolites were calculated and optimized using mono- or polyexponential regressions according to integrated equations as previously described (15).

RESULTS AND DISCUSSION

The present study was undertaken to investigate the influence of derivatization of PMEA with various prodrug moieties on total transport of PMEA across Caco-2 monolayers. Transport as well as uptake of underivatized PMEA (500 μM) in Caco-2 monolayers were shown to be negligible (both < 0.1 % over a 3 hr incubation period (12)). Ester prodrugs of PMEA with more favorable membrane permeation characteristics due to masking of the negative charges at the phosphonate group were synthesized by Shaw and Cundy (7). They selected the bis(pivaloyloxymethyl)-ester of PMEA [bis(POM)-PMEA, Figure 1] as a valuable prodrug based on promising transepithelial transport in Caco-2 monolayers.

As alternative prodrugs of PMEA, a series of carboxylesterase labile S-acyl-2-thioethyl [SATE] esters of PMEA with varying degree of lipophilicity was synthesized by Benzaria *et al.* (Figure 1) (14). These esters had been shown to be more resistant towards chemical hydrolysis in aqueous solution (buffer pH 7.2) and towards enzymatic degradation in



COMPOUND	$R_1=R_2=$
PMEA	–H
bis(POM)-PMEA	$-CH_2O-C(=O)-C(CH_3)_2-CH_3$
bis[S(methyl)ATE]-PMEA	$-(CH_2)_2S-C(=O)-CH_3$
bis[S(isopropyl)ATE]-PMEA	$-(CH_2)_2S-C(=O)-CH(CH_3)_2$
bis[S(tertbutyl)ATE]-PMEA	$-(CH_2)_2S-C(=O)-C(CH_3)_3$
bis[S(phenyl)ATE]-PMEA	$-(CH_2)_2S-C(=O)-C_6H_5$

(corresponding mono-esters: R_1=H)

Fig. 1. Chemical structures of PMEA and the prodrug esters studied. PMEA = 9-(2-phosphonylmethoxyethyl)adenine, POM = pivaloyloxymethyl and S(*X*)ATE = S(*X*)acyl-thioethyl with *X* = methyl, isopropyl, tert-butyl or phenyl.

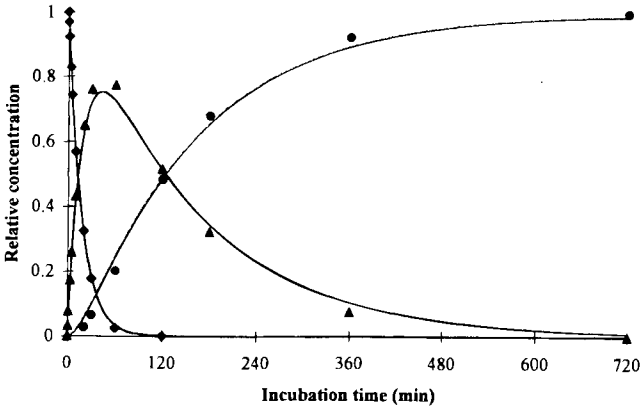


Fig. 2. Concentration-time profiles of bis(POM)-PMEA [◆], mono (POM)-PMEA [▲] and PMEA [●] after incubation of 10 μM bis (POM)-PMEA with Caco-2 homogenate. Solid lines represent computed curves obtained by mono-and polyexponential regressions according to integrated equations as described previously (15).

human serum and gastric juice as compared to bis(POM)-PMEA.

A typical concentration-time profile for the degradation of bis(POM)-PMEA in homogenate of Caco-2 monolayers is shown in Figure 2, illustrating the rapid degradation of bis(POM)-PMEA and the formation of mono(POM)-PMEA and PMEA from the prodrug. Incubation of the SATE-esters with the monolayers also resulted in the formation of the corresponding mono-ester and free PMEA. As previously reported for the bis(POM)-ester of AZT (18), bis(POM)-PMEA (12) and the bis(POM)-ester of FdUMP (19), esterases and phosphodiesterases are expected to be involved in the degradation of the SATE-esters to the mono-esters and free PMEA, respectively. During transport and uptake experiments with the SATE-esters, two additional metabolites appeared in the chromatograms, besides the formation of the corresponding mono-ester and free PMEA. As these metabolites were quantitatively important, they were further identified using LC-MS. Their structures were ascertained by comparison with the retention times, UV and mass data of authentic samples.

The additional metabolites 2-thioethyl-PMEA (M1) and its dimerisation product (M2) were detected in intracellular samples for all SATE-esters. The first metabolite (M1, Figure 3) comes from the carboxylesterase-mediated hydrolysis of the mono[SATE]-esters. Similar metabolites have been observed after incubation of SATE-esters of AZT and isoddA with CEM cell extracts (20, 15). The chemical structure of the second metabolite (M2) is also shown in Figure 3; M2 was observed by both UV and mass detection (ESI^- mode) in intracellular samples and could originate from the oxidative dimerisation of M1. The metabolite M2 was also observed in apical samples. As the metabolites M1 and M2 eventually resulted in the formation of PMEA, they were included in all further results. Complete degradation of the SATE-esters to PMEA was previously also shown in human plasma (14). No M2 could be detected during incubation of the SATE-esters with Caco-2 homogenate, suggesting that intact cell structures are required for its formation.

A broad range in sensitivity of the esters studied towards esterase-mediated hydrolysis in Caco-2 homogenate could be observed as reflected in the half-life of the esters, ranging from 4.3 ± 0.3 min for the bis[S(phenyl)ATE]-ester to 41.5 ± 0.8 min for bis[S(methyl)ATE]-PMEA. (Table I). Higher susceptibility of aromatic esters towards degradation by esterases as well as an enhanced degradation rate of more lipophilic esters has been reported previously (21,22).

As compared to the half-lives of the esters in human serum (14), different relative susceptibilities of the compounds towards esterase-mediated degradation in Caco-2 monolayers were observed. While the half-life of the bis[S(methyl)ATE]-

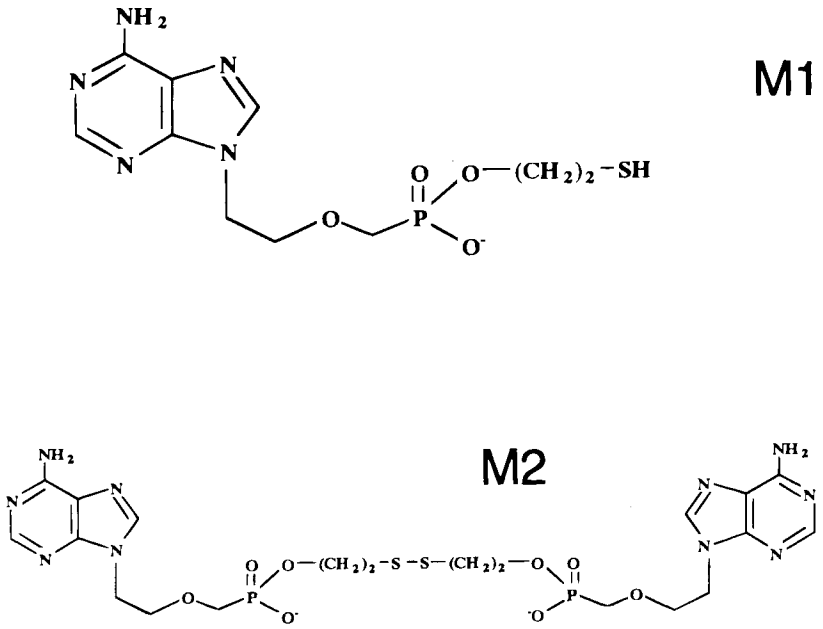


Fig. 3. Chemical structures of metabolites M1 and M2 formed during incubation of the SATE-esters with Caco-2 cell monolayers.

Table I. Half-life of the Bis-ester ($t_{1/2}$ bis), the Mono-ester ($t_{1/2}$ mono), M1 ($t_{1/2}$ M1) in Caco-2 Homogenate as Well as the Time Required for Formation of 50% PMEA ($t_{1/2\ ap}$ PMEA)

PMEA-ESTER	$t_{1/2}$ bis (min)	$t_{1/2}$ mono (min)	$t_{1/2}$ M1 (min)	$t_{1/2\ ap}$ PMEA (min)
bis(POM)-	12.5 ± 0.1	107 ± 2	—	125 ± 3
bis[S(methyl)ATE]-	41.5 ± 0.8	301 ± 2	57.6 ± 0.8	454 ± 4
bis[S(isopropyl)ATE]-	7.4 ± 0.2	81.6 ± 2	52.6 ± 4.0	170 ± 7
bis[S(tertbutyl)ATE]-	30.5 ± 0.5	242 ± 7	47.7 ± 5.0	358 ± 8
bis[S(phenyl)ATE]-	4.3 ± 0.3	1190 ± 290	—	1030 ± 300

Note: The half-lifes were calculated from rate constants which were optimized by mono-and polyexponential regressions according to integrated equations as previously described (15). Values shown are averages (± SD) of three determinations.

PMEA ester in human serum was the lowest of all SATE-esters studied, its stability in Caco-2 homogenate was highest, illustrating different substrate specificities of esterases present in Caco-2 monolayers and human serum. Half-lives of the corresponding mono-esters and M1 in Caco-2 homogenate and the time required for the conversion to 50 % PMEA are also shown in Table I. The fastest formation of PMEA was observed during incubation of the bis(POM)-ester with Caco-2 homogenate.

Transport of the various ester prodrugs across Caco-2 monolayers was studied at a concentration of 100 μM and was initiated by adding 100 μM solutions of the compounds to the apical side of the monolayers. Although concentration dependency of transport of the esters could be expected as previously shown for bis(POM)-PMEA (12), transport experiments were done at one concentration (100 μM) based on toxicity [bis(POM)-PMEA reduces cell monolayer integrity at concentrations above 100 μM], solubility {bis[S(phenyl)ATE]-PMEA solubility limited to 150 μM} and analytical considerations. Transport was assessed by measuring concentrations of the bis-esters and corresponding metabolites in the basolateral compartment. The results of transport experiments are shown in Figure 4. Large differences in total transport were observed, varying from 0.4 ± 0.1 % for bis[S(methyl)ATE]-PMEA up to 15.3 ± 0.9 % for the more lipophilic bis[S(phenyl)ATE]-ester. The latter corresponds to a more than 3-fold increase compared to the total transport of bis(POM)-PMEA (4.6 ± 0.1 %). Addition of bis(POM)-PMEA to the monolayers resulted in the largest amount of free PMEA formed at both apical and basolateral sides, which is consistent with the rapid formation of PMEA during incubation with the cell homogenates (Table I). Bis[S(tert-butyl)ATE]-PMEA was the only bis-ester of which significant amounts were observed in the basolateral compartment. Bis[S(methyl)ATE]-PMEA did not result in any significant enhancement of the total transport of PMEA. In addition, no free PMEA was found in the apical compartment after apical administration of the bis[S (methyl)ATE]-ester, which suggests intracellular localization of the enzymes responsible for the degradation of the ester prodrugs of PMEA studied. Transport studies with the SATE-esters further showed that significant amounts of M1 and its dimerisation product M2 were formed as illustrated by their presence in both apical (Table II) and basolateral (Figure 4) incubation media. Transport studies also illustrated that those esters displaying higher transport across the monolayers, were recovered to a lesser extent at the apical side at the end of the incubation period.

The lipophilicity of the esters as estimated by their log P octanol/water (14) seems to have a predictive value for total drug transport. A relationship could indeed be established between the lipophilicity of the esters and their total transport (r^2 = 0.87). This suggests that the determination of the octanol/water partition coefficient may be a valuable tool for predicting the transepithelial transport of this series of prodrug esters. However, transepithelial transport involves several consecutive processes including crossing the apical membrane, traffic through the cytosol and crossing the basolateral membrane. Therefore, it is difficult to draw simple conclusions on a relationship between lipophilicity and intestinal transport; at least partly this can be attributed to concomitant metabolism during transport; in addition, this metabolism may also depend on the structural and physicochemical characteristics of the esters, thus further compromising a relationship between total

Table II. Amounts of Bis-ester and Metabolites Remaining at the Apical (Donor) Side of Caco-2 Monolayers 3 hr After Addition of 100 μM Solutions of These Esters to the Cells

PMEA-ESTER	PMEA	M1	M2	mono-ester	bis-ester
bis(POM)-	14.3 ± 0.8	—	—	26.4 ± 0.6	19.1 ± 1.3
bis[S(methyl)ATE]-	0.4 ± 0.1	—	—	11.0 ± 0.6	85.7 ± 2.9
bis[S(isopropyl)ATE]-	2.4 ± 0.2	11.5 ± 0.7	4.5 ± 0.4	31.8 ± 1.3	12.4 ± 1.8
bis[S(tert-butyl)ATE]-	0.8 ± 0.1	4.0 ± 0.2	1.6 ± 0.2	14.1 ± 0.6	12.5 ± 1.4
bis[S(phenyl)ATE]-	1.6 ± 0.1	6.7 ± 0.3	3.7 ± 0.3	12.6 ± 1.3	2.2 ± 0.3

Note: Values represent percentages (± SD) of amounts initially added to the cells (n = 3).

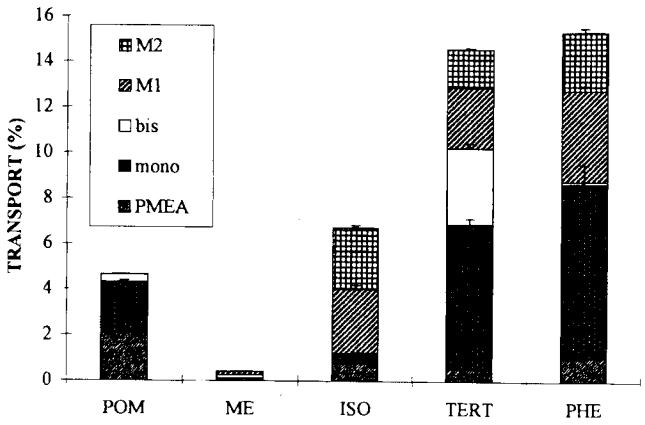


Fig. 4. Total cumulative transport (+ SD) of esters of PMEA across Caco-2 monolayers for a 3 hr incubation period (n = 5). All data are expressed as a percentage of the amount of bis-ester initially added to the apical side. ['POM' = bis(POM)-PMEA, 'ME' = bis[S(methyl) ATE]-PMEA, 'ISO' = bis[S(isopropyl)ATE]-PMEA, 'TERT' = bis[S(tert-butyl)ATE]-PMEA, 'PHE' = bis[S(phenyl)ATE]-PMEA; 'bis' = the bis-ester, 'mono' = the mono-ester, for M1 and M2 see Figure 3].

transport and lipophilicity. In this context, the use of the Caco-2 model has the advantage of being a rapid and comprehensive test system for the evaluation of selected compounds developed as potentially orally active drugs, since this *in vitro* model allows to study the combined effect of membrane permeation and enzymatic stability.

Results of uptake experiments are shown in Figure 5; these studies revealed that after uptake of the SATE-esters (100 μM), the metabolites M1 and M2 were quantitatively even more important than PMEA; PMEA was found to be the main intracellular metabolite after uptake of bis(POM)-PMEA. Total intracellular concentrations (180 min) of the metabolites were calculated based on an estimated cell height of 30 μm (23) and amounted to 3.3 mM for bis(POM)-PMEA and bis[S(phenyl)

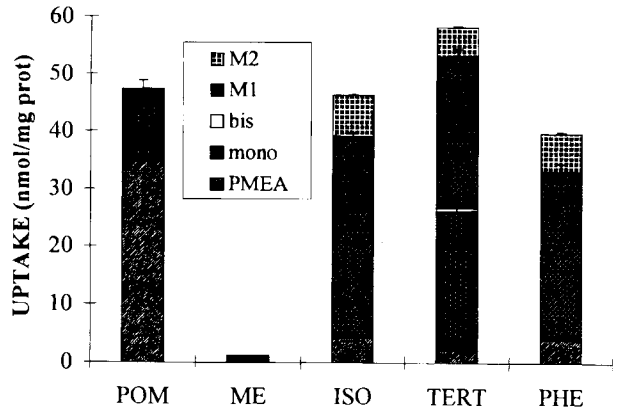


Fig. 5. Uptake of the esters of PMEA in Caco-2 monolayers, represented as cumulative intracellular amounts (+ SD) of esters and metabolites after a 3 hr incubation period (n = 3) and expressed as nmol per mg protein. ['POM' = bis(POM)-PMEA, 'ME' = bis[S(methyl) ATE]-PMEA, 'ISO' = bis[S(isopropyl)ATE]-PMEA, 'TERT' = bis[S(tert-butyl)ATE]-PMEA, 'PHE' = bis[S(phenyl)ATE]-PMEA; 'bis' = the bis-ester, 'mono' = the mono-ester, for M1 and M2 see Figure 3].

ATE]-PMEA, and to 3.6 mM and 4.3 mM for bis[S(isopropyl)ATE]-PMEA and bis[S(tert-butyl)ATE]-PMEA, respectively. Although intracellular levels reached for M1 were much more elevated than for M2, the relatively high concentrations of M2 in the apical compartment could be explained by the more lipophilic nature of the latter, which could favor the release of this intracellularly formed metabolite. Figure 5 also illustrates that the bis[S(tert-butyl)ATE]-ester was the only bis-ester which (albeit at a low concentration) could be observed intracellularly after a 3 hr incubation period. This is consistent with the relatively high resistance of this ester towards enzyme-mediated degradation in Caco-2 homogenate.

The antiviral effect of PMEA can be attributed to the formation of diphosphorylated PMEA (PMEApp) as its active metabolite. Using ion-exchange chromatography, allowing the determination of PMEA, PMEAp and PMEApp, the intracellular presence of PMEA together with PMEApp was confirmed after uptake of bis(POM)-PMEA, suggesting further intracellular metabolism of PMEA towards PMEApp in Caco-2 monolayers. The intracellular level of PMEApp amounted to ~25% of the PMEA concentration after 120 min incubation with 100 μM bis(POM)-PMEA (data not shown). The involvement of AMP kinase has been suggested for the conversion of PMEA to PMEApp (24); this enzyme converts PMEA to PMEApp in two steps, with the formation of PMEAp as the intermediate metabolite. As the latter was not detected inside the Caco-2 cells, an alternative metabolic pathway mediated by 5-phosphoribosyl-1-pyrophosphate (PRPP) synthetase may also be suggested for the formation of PMEApp from PMEA in Caco-2 monolayers. This enzyme has previously been reported to be involved in the direct conversion of PMEA to PMEApp (25).

We conclude that some of the SATE-esters of PMEA studied can be considered as promising candidate prodrugs in the development of an orally available prodrug of PMEA: the total flux across Caco-2 monolayers of the three most lipophilic esters is significantly increased as compared to bis(POM)-PMEA. Additional studies including the investigation of the first-pass metabolism and the stability of the esters in plasma, are required to further explore the potential of the SATE-esters for the enhancement of the oral bioavailability of PMEA. In addition, as an increased lipophilicity is often associated with a compromised aqueous solubility and dissolution rate, the combined effect of all these physicochemical factors affecting intestinal absorption has to be assessed.

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