**INTESTINAL ABSORPTION ENHANCEMENT OF THE ESTER PRODRUG TENOFOVIR DISOPROXIL FUMARATE THROUGH MODULATION OF THE BIOCHEMICAL BARRIER BY DEFINED ESTER MIXTURES**

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**ABSTRACT:**

The effect of discrete esters and ester mixtures on the intestinal stability and absorption of tenofovir disoproxil fumarate (tenofovir DF, an esterase-sensitive prodrug of the antiviral tenofovir) was compared with the effect of strawberry extract, which has been shown to enhance the absorption of the prodrug across Caco-2 monolayers and in rat ileum. In addition, the mechanism of absorption enhancement was investigated. In rat intestinal homogenates, complete inhibition of the conversion of tenofovir DF (as obtained by strawberry extract) could only be obtained at relatively high concentrations of the discrete esters or by using mixtures of esters (e.g., propyl p-hydroxybenzoate 0.02%, octyl acetate 0.02%, ethyl caprylate 0.01%). Coincubation of tenofovir DF with this mixture also resulted in an enhancement of its absorption in the in vitro Caco-2 system as well as in rat ileum. As tenofovir DF is a substrate for P-glycoprotein (P-gp)-related efflux carriers in the Caco-2 model, the modulatory effect of the ester mixtures was studied on the functionality of P-gp using cyclosporin A (CsA) as a model substrate. Strawberry extract as well as the mixture of three esters interfered with the absorptive transport of CsA across Caco-2 monolayers, illustrating that both mixtures interfere with both esterase-activity and P-gp functionality. This concerted barrier was not observed in rat ileum, suggesting differential functional activities of the biochemical barrier toward tenofovir DF in different absorption systems. Overall, our results illustrate that modulation of the biochemical barrier (metabolism and efflux) of tenofovir DF by ester mixtures can be used to increase the intestinal absorption of tenofovir DF in an in vitro and in situ absorption model; the mechanism of action appears to be a complex interplay of different systems; the differential expression of carriers and enzymes in different systems illustrates the difficulty of extrapolating observations between different systems/species.

Ester prodrugs are commonly used to increase the intestinal absorption of drugs with permeability-limited absorption. The high functional activity of esterases in blood and liver allows a rapid bioactivation of the prodrug after reaching the systemic circulation (Satoh and Hosokawa, 1998). However, the increased efficiency of prodrugs to pass the intestinal barrier may be decreased by its rapid esterase-mediated hydrolysis at the level of the intestinal mucosa, offsetting the diffusion of the lipophilic, intact esters across the epithelial monolayer. This phenomenon primarily occurs at the level of the mucosa of small intestine, due to its high esterase activity (Yoshigae et al., 1998). Two examples of the failure of the intestinal absorption of tenofovir (probably mediated by phosphodiesterases) at the level of the intestinal mucosa may be an important barrier limiting the trans-epithelial transport of tenofovir DF (Naesens et al., 1998) (Scheme 1).

Recently, we demonstrated that coincubation with nature-identical strawberry extract enhances the efficiency of the prodrug to increase the intestinal absorption of tenofovir DF (Naesens et al., 1998) (Scheme 1). We further showed that coincubation with strawberry extract resulted in complete inhibition of the conversion of tenofovir DF to its (mono)ester-equivalent (mediated by carboxylesterases) and further to tenofovir (mediated by phosphodiesterases) at the level of the intestinal mucosa may be an important barrier limiting the trans-epithelial transport of tenofovir DF (Scheme 1). These results in the Caco-2 model; the mechanism of action appears to be a complex interplay of different systems; the differential expression of carriers and enzymes in different systems illustrates the difficulty of extrapolating observations between different systems/species.

Tenofovir disoproxil fumarate [tenofovir DF1, formerly known as bis(POC)-PMPA] is a bis-ester prodrug of the acyclic nucleoside phosphonate tenofovir. Tenofovir has a strong activity against human immunodeficiency virus infection in humans (Deeks et al., 1998). However, it is characterized by a permeability-limited oral absorption due to its hydrophilic nature (Shaw et al., 1997). In the prodrug, the two negative charges of tenofovir are masked by isopropylxycarbonyloxymethyl moieties, which increase the lipophilicity of the compound and thus its permeation across membranes. Based on its favorable pharmacokinetic profile compared with other ester prodrugs, tenofovir DF was selected as an orally active form of tenofovir (Arimilli et al., 1997). It was recently approved by the Food and Drug Administration for the treatment of human immunodeficiency virus infection (Viread).

It was shown that the degradation of tenofovir DF to its (mono)ester-equivalent (mediated by carboxylesterases) and further to tenofovir (probably mediated by phosphodiesterases) at the level of the intestinal mucosa may be an important barrier limiting the trans-epithelial transport of tenofovir DF (Naesens et al., 1998) (Scheme 1). Recently, we demonstrated that coincubation with nature-identical strawberry extract enhances the efficiency of the prodrug to increase the intestinal absorption of tenofovir DF in an in vitro and in situ absorption model; the mechanism of action appears to be a complex interplay of different systems; the differential expression of carriers and enzymes in different systems illustrates the difficulty of extrapolating observations between different systems/species.

**ABBREVIATIONS USED ARE:** DF, disoproxil fumarate; P-gp, P-glycoprotein; TM, transport medium; EM, ester mixture.
the intestinal absorption of tenofovir across Caco-2 monolayers (Van Gelder et al., 1999) as well as in rat ileum (Van Gelder et al., 2000a). Among a multitude of other substances, fruit extracts contain several flavoring esters. It was postulated that inhibition of the metabolism of tenofovir DF by these esters and/or by other compounds could at least partially explain the absorption enhancement observed in the presence of the fruit extract. The limiting factor of strawberry extract in view of its incorporation in a pharmaceutical formulation is that, besides a series of esters, the extract contains a broad variety of other compounds, which makes it difficult to control its absorption enhancing effect. The aim of this study was to reduce the complexity of the mixture and, thereby, to ease the clinical applicability of this absorption enhancing approach. We therefore investigated the effects of discrete esters as well as that of defined mixtures of esters on the metabolism and intestinal transport of tenofovir DF using homogenates from rat small intestine, the in vitro Caco-2 model and in situ perfusion of rat intestine.

Besides esterase-mediated conversion, a second barrier that may limit the absorption of tenofovir DF appears to be a P-glycoprotein (P-gp)-related efflux mechanism. We therefore explored whether interference with P-gp may be involved in the absorption enhancing effect of strawberry extract and of the ester mixtures. It was recently shown that several fruit or plant extracts interfere with this efflux mechanism at the level of the gut (i.e., P-gp). A few examples from the literature include the inhibition of P-gp by orange juice (Takanaga et al., 2000), rosemary extract (Plouzek et al., 1999), St. John’s Wort extract (Troutman et al., 2000), and grapefruit juice (Takanaga et al., 1998; Spahn-Langguth and Langguth, 2001). To explore a possible interference of the ester mixtures with the functional P-gp efflux activity, their effect was studied on the polarity in transport of the P-gp model substrate cyclosporin A across Caco-2 monolayers (Augustijns et al., 1993).

### Materials and Methods

#### Chemicals

Tenofovir [(R)-PMPA] and tenofovir DF were obtained from Gilead Sciences Inc. (Foster City, CA). Transport medium (TM) consisted of Hanks’ balanced salt solution supplemented with 10 mM Hepes, adjusted to a pH of 7.4. TM used in Caco-2 transport experiments was further supplemented up to 25 mM glucose. Strawberry extract was provided by Givaudan-Roure (Dortmund, Germany). Phenyl benzoate, phenethyl isobutyrate, p-tolyl benzoate, phenethyl acetate, ethyl benzoate, methylphenyl acetate, ethyl caproate, ethyl propionate, methyl acetate, ethyl formate, and phenyl acetate were purchased from Sigma-Aldrich (Bornem, Belgium), Octyl acetate, hexyl acetate, methyl anthranilate, and isopropyl acetate were obtained from Acros Organics N.V. (Geel, Belgium), whereas decyl acetate was obtained from ICN Pharmaceuticals NV/SA (Asse-Relegem, Belgium). Merck-Belgolab (Overijse, Belgium) provided us with isooamyl butyrate, isooamyl acetate, butyl acetate, ethyl butyrate, propyl acetate, and isobutyl acetate. Ethyl acetate was purchased from Carlo Erba Reagents (Milan, Italy). Methyl, ethyl, and propyl paraben as well as pipamipicillin, bacampicillin, and talampicillin were kindly provided by Professor J. Hoogmartens (Laboratorium voor Farmaceutische Chemie, KULeuven, Leuven, Belgium). Cyclosporin A was a kind gift of Dr. T. Bradshaw (GlaxoSmithKline, Research Triangle Park, North Carolina), whereas [3H]cyclosporin A was obtained from Amersham Biosciences (Ghent, Belgium). All other chemicals were of reagent grade and the highest purity commercially available.

#### Preparation of Homogenates from Rat Intestine

Homogenates from rat intestine were prepared as previously described (Van Gelder et al., 2000b). The intestinal mucosa was removed by scraping the intestine with a glass microscope slide. The scraped were homogenized at 0°C in 5 ml of cold TM using a Potter-Elvehjem tube and pestle. After centrifugation of the crude intestinal tissue homogenates at 10,000 g for 10 min, the supernatants were harvested and kept at 0°C. Prior to freezing, protein content was determined according to the method of Lowry et al. (1951) using bovine serum albumin as a standard. The homogenate was aliquoted and frozen at −40°C until use. Freezing of the homogenate did not alter the degradation rate of tenofovir DF under the conditions used.

#### Degradation Studies with Tenofovir DF in Intestinal Homogenates

Metabolism studies were performed under linear conditions at 37°C in the presence or absence of esters at a concentration of tenofovir DF between 25 and 400 μM in intestinal homogenates at 0.05 mg of protein/ml. The conversion of tenofovir DF was initiated by spiking a solution of tenofovir DF with the intestinal homogenate in the absence or presence of esters. In a second set of experiments, ester mixtures were preincubated with intestinal homogenate (0–60 min) before starting the incubation with tenofovir DF, to assess the enzymatical stability of the ester mixtures. After 5 min, the reaction was stopped by adding 100 μl of the incubation solution to 900 μl of ice-cold methanol. After centrifugation at 10,000g (10 min, 4°C), 800 μl of the supernatant was evaporated by a gentle stream of air and redissolved in 800 μl of TM adjusted to pH 3.3 with HCl 0.05 N; 50 μl was injected into the high-performance liquid chromatography system. Samples were analyzed using a previously described method (Van Gelder et al., 1999). Calculated values of log P of the discrete esters were obtained at http://www.acdlabs.com/ilab. The calculation was based on an algorithm that uses a database containing one or more experimental log P values for over 3,600 structures with 500 different functional groups. Calculated values generally have an accuracy of ±0.3 log P units.

#### Transepithelial Transport Studies with Tenofovir DF across Caco-2 Monolayers

Caco-2 cells were cultured as previously described (Augustijns et al., 1998). For the determination of the transepithelial flux of tenofovir DF across Caco-2 monolayers, the polarized monolayers were preincubated with TM for 30 min with or without esters, after which trans-epithelial electrical resistance values were measured to check cell monolayer integrity. The medium was then replaced by TM with tenofovir DF (100 μM) at the donor side in the presence or absence of esters. Samples were taken from the receiver compartment after 60 min. Samples were adjusted with HCl 0.05 M to pH 3.3 and kept at 4°C to reduce chemical degradation of tenofovir DF during storage. Unless stated otherwise, all flux experiments were conducted in triplicate. Transport is expressed as absolute amounts of tenofovir DF and metabolites appearing at the receiver side.

#### Transepithelial Transport Studies with [3H]Cyclosporin A across Caco-2 Monolayers

The influence of strawberry extract and of ester mixtures on the polarity in transport of [3H]cyclosporin A was studied as follows. After a preincubation of 30 min in the presence or absence of esters or strawberry extract at the apical side, transport was initiated by adding [3H]cyclosporin A (0.1 μg) and a fixed amount of unlabeled cyclosporin A to the donor compartment to a final concentration of 1 μM in the presence or absence of...
defined ester mixtures or strawberry extract at the apical side. Transport was assayed in both directions. Following incubation (60 min), the samples in the acceptor compartment were removed by multiple pipetting and placed (along with the tips) in scintillation vials (16 ml of scintillation liquid, Ready Safe; Beckman Instruments, Fullerton, CA) for liquid scintillation counting (Liquid Scintillation Counter, Wallac 1410; Beckmann Instruments). All solutions of cyclosporin A were made in siliconized glass tubes to avoid its adsorption. At the end of the experiment, the insert was withdrawn, and 10 μl of a 1 mM unlabeled cyclosporin A solution and 200 μl of dimethyl sulfoxide were added to the wells for 1 h before collecting the samples to assure total recovery of [3H]cyclosporin A in the basolateral acceptor compartment.

In Situ Rat Intestinal Perfusion Studies with Tenofovir DF. In situ perfusion experiments were performed based on a previously described method (Annaert et al., 2000, Van Gelder et al., 2000a). Male Wistar rats (± 300 g, Animalium, K. U. Leuven, Leuven, Belgium) were used. Before the start of the experiment, the intestinal segment was perfused for 15 min with TM in the presence or absence of ester mixtures. The flow rate of the perfusate amounted to 3 ml/min. The intestinal segment was perfused with tenofovir DF (0.1 mM) in the presence or absence of ester mixtures. Blood from the mesenteric vein was collected in heparinized tubes over 5-min time intervals for 30 min. Fresh rat blood was administered through the jugular vein (0.5 ml/min) to compensate for the blood loss from sampling. In addition, samples were taken from the perfusion medium in the middle of each time interval. Blood samples were centrifuged at 5,000g for 10 min (4°C), and the obtained plasma fractions were weighed. As no significant accumulation of tenofovir DF nor its metabolites occurred in red blood cells (<1.5% of the total amount in blood), concentrations of tenofovir and tenofovir (mono)ester were determined in plasma instead of total blood. Tenofovir and tenofovir (mono)ester-prodrug concentrations in plasma were determined according to a previously described method (Van Gelder et al., 2000a). Results are expressed as tenofovir-equivalents (i.e., the sum of the different metabolites of tenofovir DF) appearing in mesenteric plasma. Perfusion samples were tested for lactate dehydrogenase release to measure cellular membrane damage using the LD-L 20 kit (Sigma Diagnostics NV/SA, Bornem, Belgium).

Statistical Analysis. Data are expressed as average value ± S.D. (n = 3), unless stated otherwise. Student’s t test was performed to compare two data sets. A p value of <0.05 was considered significant.

Results

The effect of discrete ester compounds (1 mM) on the metabolism of tenofovir DF (0.025 mM) in homogenates from rat intestine is shown in Table 1. Mass balance was maintained in all experiments. Recovery of tenofovir DF and its (mono)ester after 5 min amounted to 100.8 ± 0.4% of the initial amounts of tenofovir DF and metabolite.

<table>
<thead>
<tr>
<th>Name</th>
<th>Calculated log P</th>
<th>Inhibition</th>
<th>Name</th>
<th>Calculated log P</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strawberry extract</td>
<td>3.59</td>
<td>99.0 ± 3.0</td>
<td>Aspirin</td>
<td>1.19</td>
<td>35.5 ± 6.0</td>
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<tr>
<td>Phenyl benzoate</td>
<td>2.93</td>
<td>94.5 ± 3.8</td>
<td>Methyl anthranilate</td>
<td>2.04</td>
<td>32.0 ± 3.7</td>
</tr>
<tr>
<td>Propyl paraben</td>
<td>3.18</td>
<td>92.3 ± 1.3</td>
<td>Ethyl caproate</td>
<td>2.83</td>
<td>27.1 ± 8.8</td>
</tr>
<tr>
<td>Phenethyl isobutyrate</td>
<td>1.91</td>
<td>92.1 ± 11.7</td>
<td>Decyl acetic</td>
<td>4.96</td>
<td>37.0 ± 5.4</td>
</tr>
<tr>
<td>Bacampicillin</td>
<td>2.53</td>
<td>92.1 ± 1.4</td>
<td>Isoamyl butyrate</td>
<td>3.18</td>
<td>16.9 ± 9.8</td>
</tr>
<tr>
<td>Talampicillin</td>
<td>4.05</td>
<td>90.0 ± 4.1</td>
<td>Isoamyl acetate</td>
<td>2.12</td>
<td>12.1 ± 6.7</td>
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<tr>
<td>p-Tolyl benzoate</td>
<td>4.05</td>
<td>76.0 ± 4.6</td>
<td>Butyl acetic</td>
<td>1.77</td>
<td>12.1 ± 3.4</td>
</tr>
<tr>
<td>Ethyl paraben</td>
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<td>62.9 ± 6.2</td>
<td>Ethyl butyrate</td>
<td>1.77</td>
<td>11.7 ± 4.8</td>
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<td>Diethyl phthalate</td>
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<td>Octyl acetate</td>
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<td>Ethyl caprylate</td>
<td>3.90</td>
<td>58.2 ± 2.5</td>
<td>Methyl acetate</td>
<td>0.18</td>
<td>9.6 ± 3.3</td>
</tr>
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<td>Methyl paraben</td>
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<td>57.0 ± 12.9</td>
<td>Ethyl acetate</td>
<td>0.71</td>
<td>9.1 ± 6.5</td>
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<td>Phenethyl acetate</td>
<td>2.30</td>
<td>52.7 ± 7.0</td>
<td>Ethyl formate</td>
<td>0.30</td>
<td>8.3 ± 7.7</td>
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<td>Ethyl benzoate</td>
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<td>47.1 ± 4.0</td>
<td>Phenyl acetate</td>
<td>1.56</td>
<td>6.8 ± 7.9</td>
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<tr>
<td>Hexyl acetate</td>
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<td>44.3 ± 8.7</td>
<td>Isobutyl acetate</td>
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<td>6.6 ± 3.3</td>
</tr>
<tr>
<td>Methyl phenylacetate</td>
<td>1.93</td>
<td>38.2 ± 8.0</td>
<td>Isopropyl acetate</td>
<td>1.06</td>
<td>2.4 ± 3.7</td>
</tr>
</tbody>
</table>

Fig. 1. Influence of lipophilicity of discrete esters on the degree of inhibition of the metabolism of tenofovir DF (25 μM) in rat intestinal homogenates; values represent the percentage of inhibition of tenofovir (mono)ester formed as a function of the calculated log P (mean ± S.D.; n = 3).

In the absence of esters, the percentage of (mono)ester formed after 5 min amounted to 26.1 ± 3.3% of the initial amount of tenofovir DF. No tenofovir was formed after 5 min. Strawberry extract (1% v/v) completely inhibited the enzymatic conversion of the prodrug to the (mono)ester. The effect of the discrete esters on the metabolism of tenofovir DF ranged from a negligible effect to almost complete inhibition. No correlation was observed when the effect of the esters was plotted versus their calculated lipophilicity (Fig. 1).

For several esters, a concentration-dependent inhibitory effect on the metabolism of tenofovir DF was observed, as illustrated for propyl paraben in Fig. 2. Similar observations were made for other esters (data not shown). The Lineweaver-Burk plot for the metabolism of tenofovir DF (0.025–0.4 mM) yielded a K_{max} value of 0.0232 mM and a V_{max} of 0.80 nmol/s · mg of protein. V_{max} was not influenced by the addition of 0.5 mM propyl paraben (0.78 nmol/s · mg of protein), which is consistent with a competitive inhibition mechanism of the conversion of tenofovir DF by propyl paraben.

The effect of ester hydrolysis products on the enzymatic conversion of tenofovir DF was also investigated. For this purpose, octanol and acetic acid were selected as model compounds; inclusion of octyl acetate resulted in 62% inhibition of the metabolism of tenofovir DF (Table 1). No inhibitory effect on the conversion of the prodrug was

TABLE 1

Influence of discrete esters on the metabolism of tenofovir DF in homogenates from rat intestine; the concentration used was 1 mM for the esters and 1% v/v for the strawberry extract; values represent the percentage of inhibition of the enzymatic conversion of the prodrug to the (mono)ester after 5 min of incubation (mean ± S.D., n = 3)
observed with the respective hydrolysis products at a concentration of 100 μM. The pH of the incubation medium remained stable for at least 2 h when esters were included in intestinal homogenates, indicating that the inhibitory effect of the esters is not caused by a shift in pH of the incubation medium and that the amount of acidic hydrolysis products formed was not sufficient to counteract the buffer capacity of the incubation medium. Overall, these data suggest that the inhibitory effect can be attributed to esters and not to an effect of possible hydrolysis products.

In a next step, several ester mixtures (total concentration of esters up to approximately 2.5 mM or 0.05% v/v) were prepared and evaluated for their inhibitory effect on the metabolism of tenofovir DF. The composition of these ester mixtures is shown in Table 2. The mixtures were selected based on the inhibitory effect of the individual esters. Both ester mixtures (EM1 and EM2) completely inhibited the enzymatic degradation of tenofovir DF in homogenates from rat intestine at a protein content of 0.05 mg of protein/ml (Table 2). At higher protein concentrations, EM2 appeared to have a lower inhibitory effect as compared with EM1 and strawberry extract (data not shown). In addition, the inhibitory capacity of EM2 decreased as a function of contact time with the intestinal homogenate, suggesting a gradual degradation of the esters present in EM2 (Fig. 3). When a similar experiment was performed with EM1, its inhibitory capacity was maintained after 60 min of contact time of the esters with the homogenate. These data illustrate the higher inhibitory capacity of EM1 as compared with EM2.

We next examined the effect of the ester mixtures on the apical-to-basolateral transport of tenofovir DF (0.1 mM) across Caco-2 monolayers (Table 3). Coincubation with EM1 resulted in an increase in tenofovir equivalents appearing at the basolateral side; the effect of EM2 on the absorptive transport of tenofovir DF was determined in a different test set (Table 3); a relatively high interbatch variability between the reference experiments was obtained, which illustrates the importance of comparing data within one batch. EM2 did not enhance the appearance of tenofovir equivalents at the basolateral side, when tenofovir DF was coincubated with the mixture at the apical chamber.

The influence of the ester mixtures or of strawberry extract on the P-gp functional activity in the Caco-2 system was evaluated in a next set of experiments. Transport polarity of tenofovir DF as well as the leveling effect of cyclosporin A (10 μM) on transport polarity across Caco-2 monolayers indicate that tenofovir DF is a substrate of a P-gp-related efflux pump in the Caco-2 system (Fig. 4). Cyclosporin A did not inhibit the conversion of tenofovir DF in homogenates from rat intestine (data not shown), suggesting a specific interference of the immunosuppressive agent on P-gp-related efflux of tenofovir DF. The increase in absorptive transport of tenofovir equivalents observed upon inclusion of cyclosporin A was further enhanced by adding EM1 to the incubation medium (Table 3A), indicating that both esterase-mediated conversion and efflux compromise the absorption of tenofovir DF.

To study whether strawberry extract and the ester mixtures interfere with the functional activity of P-gp, cyclosporin A was selected as a model substrate; as the transport of tenofovir DF is simultaneously influenced by metabolism and efflux, it complicates the determination of a specific modulation of strawberry extract and the ester mixtures on the P-gp functional activity of Caco-2 monolayers. Progesterone, which was used as a positive control for P-gp inhibition, almost completely abolished the transport polarity of cyclosporin A (Fig. 5).
Strawberry extract also significantly increased the absorptive transport \((p < 0.01)\) and decreased the secretory transport \((p < 0.01)\) of cyclosporin A, indicating an interference of the extract with P-gp activity. Although no effect on secretory transport could be observed \((p > 0.05)\), the enhancement in absorptive transport of cyclosporin A \((p < 0.05)\) suggests that also EM1 has an effect on P-gp functional activity in Caco-2 cells (Fig. 5a). In contrast to EM1 and strawberry extract, EM2 did not interfere with the transepithelial transport of cyclosporin A \((p > 0.05)\) across Caco-2 monolayers (Fig. 5b).

We next performed in situ perfusion studies to assess the influence of EM1 and EM2 on the appearance of tenofovir equivalents in mesenteric plasma during a perfusion of rat ileum with tenofovir DF (100 μM), in comparison with our data previously obtained with strawberry extract (Van Gelder et al., 2000a). With EM1, a similar increase in appearance of tenofovir equivalents was observed as with strawberry extract (Fig. 6). Coperfusion of tenofovir DF in rat ileum with EM2 did not result in an increase in appearance of tenofovir equivalents. Neither EM1 nor EM2 affected membrane integrity, as illustrated by the absence of an increase in lactate dehydrogenase activity in perfusate compared with a perfusion in the absence of ester mixture. We also tested the effect of a P-gp inhibitor on the appearance of tenofovir-equivalents in rat ileum. When tenofovir DF was coperfused with cyclosporin A (10 μM) in rat ileum, no significant increase in tenofovir-equivalents in mesenteric plasma was observed \((6.51 ± 1.43 \text{ pmol} \cdot \text{cm}^{-1} \cdot \text{min}^{-1})\). Tenofovir equivalents appearing when tenofovir DF was perfused in the absence of cyclosporin A compared with 7.37 ± 2.36 pmol \cdot cm^{-1} \cdot min^{-1} \text{ in the presence of P-gp inhibitor).}

### Discussion

This study focuses on the applicability of coadministration of selected ester compounds with tenofovir DF to enhance the absorption of the prodrug. Previous studies have shown that inhibition of conversion of tenofovir DF by strawberry extract resulted in an increased

### Table 3

<table>
<thead>
<tr>
<th>Tenofovir</th>
<th>Tenofovir Mono(ester)</th>
<th>Tenofovir DF</th>
<th>Tenofovir Equivalents</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Control</td>
<td>0.30 ± 0.06</td>
<td>0.21 ± 0.02</td>
<td>0.51 ± 0.05</td>
</tr>
<tr>
<td>EM 1</td>
<td>0.22 ± 0.04</td>
<td>1.09 ± 0.05</td>
<td>1.31 ± 0.08</td>
</tr>
<tr>
<td>CsA</td>
<td>0.74 ± 0.06</td>
<td>0.66 ± 0.03</td>
<td>1.40 ± 0.09</td>
</tr>
<tr>
<td>CsA + EM 1</td>
<td>0.43 ± 0.03</td>
<td>2.81 ± 0.03</td>
<td>3.24 ± 0.01</td>
</tr>
<tr>
<td>B Control</td>
<td>0.20 ± 0.05</td>
<td>0.11 ± 0.04</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td>EM 2</td>
<td>0.22 ± 0.09</td>
<td>0.15 ± 0.02</td>
<td>0.37 ± 0.08</td>
</tr>
</tbody>
</table>

Influence of EM1, EM2, and strawberry extract 1% (v/v) on the appearance of tenofovir DF and metabolites in the basolateral compartment of Caco-2 monolayers after adding tenofovir DF (100 μM) at the apical side; values represent the amounts (nanomoles) appearing in the receiver chamber after 60 min \((\text{mean} \pm \text{S.D.}, n = 3)\). Separate experiments (A and B) performed on different days.
absorption of the prodrug (Van Gelder et al., 1999). To standardize the effect of strawberry extract, discrete esters and mixtures of esters were selected and evaluated for their effect on the conversion and transepithelial transport of the prodrug. In a first step, the effect of the esters on the conversion of tenofovir DF was assessed using homogenates from rat intestine (Table 1). Our results indicate that a minimal lipophilicity (log P > 2) is required to obtain an inhibitory effect of the ester on the conversion of tenofovir DF. Within a series of structurally related esters (acetate esters), esters with higher log P values (log P > 4; e.g., decyl acetate) had a lower degree of inhibition of the metabolism of tenofovir DF compared with esters with log P values around 3 (e.g., hexyl acetate, octyl acetate). Although an effect of chain length and lipophilicity within a homologous series of esters has previously been described (Chang and Lee, 1982), it is obvious that other factors are involved, including solubility and parameters which determine the affinity of an ester for the active pocket of carboxylesterases [e.g., size, steric hindrance, hydrogen bonding potential and charge (Durrer et al., 1992; Foroutan and Watson, 1999)].

This is supported by the observation that no correlation was found between lipophilicity of all esters tested (independent on the chemical structure) and their inhibitory effect on the metabolism of tenofovir DF (Fig. 1).

The data above illustrate that inhibition of tenofovir DF metabolism may indeed be obtained by coinucubation of tenofovir DF with diverse esters in rat intestinal homogenates; however, relatively high concentrations of the inhibitory esters were required. In addition, parallel studies had indicated that transport of the prodrug across Caco-2 monolayers was not enhanced by discrete esters. Therefore, in a next step, we determined the effect of standardized EMs on the metabolism of the prodrug. EM1 is a mixture of three esters at relatively high concentrations, whereas EM2 is a mixture of 15 esters at relatively low concentrations (Table 2). Both ester mixtures inhibited the metabolism of tenofovir DF in homogenates from rat intestine in a similar way as did strawberry extract under the conditions stated in Table 2. However, EM2 had a lower inhibitory capacity compared with EM1 or strawberry extract at higher protein concentrations (data not shown). In addition, the esters of EM2 were prone to enzymatic degradation (Fig. 3). EM1, on the other hand, showed a similar inhibitory capacity on the enzymatic conversion of tenofovir DF as strawberry extract in all conditions tested.

In a next step, the effect of the ester mixtures was tested on the transepithelial transport of the prodrug across Caco-2 monolayers, in comparison to our previous data, indicating a 3-fold increase in transepithelial transport across Caco-2 monolayers when tenofovir DF was coincubated with strawberry extract (Van Gelder et al., 1999). Coincubation of tenofovir DF with EM1 resulted in an enhanced transport of tenofovir equivalents across Caco-2 monolayers, mainly due to an increase of intact prodrug appearing at the basolateral side. This indicates an inhibition of the conversion of tenofovir DF during transepithelial transport by the ester mixture. Coincubation with EM2 failed to alter the relative amounts of intact prodrug appearing at the receiver chamber, which may be due to the lower inhibitory capacity of EM2 compared with EM1.

In addition to esterase-mediated conversion of tenofovir DF during transepithelial transport, the effect of another biochemical absorption barrier (i.e., P-gp-mediated efflux) on the transport of the prodrug was assessed in the Caco-2 system. Several authors have recently pointed out the impact of natural extracts on P-gp-mediated efflux (Plouzek et al., 1999; Takanaga et al., 2000; Spahn-Langguth and Langguth, 2001). Our results show that the transport of the lipophilic ester prodrug tenofovir DF is modulated by a P-gp-related efflux mechanism in the Caco-2 system; the P-gp inhibitor cyclosporin A almost completely abolished the transport polarity of tenofovir DF across Caco-2 monolayers (Fig. 4). These findings suggest that P-gp-related efflux may be a second barrier system limiting the transepithelial transport of tenofovir DF. A combination of EM1 and a P-gp inhibitor (cyclosporin A) resulted in a further enhanced transport of tenofovir DF in comparison to EM1 or cyclosporin A alone, indicating that esterase-mediated conversion and P-gp-related efflux form a concerted barrier to the transport of the prodrug across Caco-2 monolayers. To evaluate whether strawberry extract and/or the ester mixtures interfere with P-gp, their effect was studied on the transport polarity of cyclosporin A across Caco-2 monolayers. In all experiments, strawberry extract significantly increased the absorptive transport of cyclosporin A (Fig. 5, a and b), suggesting that one or more until now unknown compounds present in strawberry extract may also interfere with the P-gp-related efflux of tenofovir DF. EM1 only significantly affected the absorptive transport of cyclosporin A. These data suggest a significant but lower interference compared with strawberry extract of EM1 with P-gp in the Caco-2 system. In contrast to EM1, EM2 did not interfere with the transepithelial transport of cyclosporin A. Strawberry extract and, to a lesser extent EM1, thus appear to simultaneously interfere with the concerted barrier (esterase-mediated degradation and P-gp-related efflux) to the transepithelial transport of tenofovir DF in the Caco-2 system.

Besides the in vitro Caco-2 system, a second absorption model was used to study the effect of ester mixtures on the intestinal absorption of tenofovir DF. Studies with the ester mixtures in a perfusion of rat ileum with tenofovir DF revealed that EM1, a mixture with a similar inhibitory capacity on the conversion of tenofovir DF in intestinal homogenates as compared with strawberry extract, increased the appearance of tenofovir equivalents in mesenteric plasma in a similar way as did strawberry extract, while no effect was observed on the appearance of tenofovir equivalents in the presence of EM2 (Fig. 6). In contrast to the Caco-2 system, no interference of a P-gp-related efflux mechanism could be demonstrated in rat ileum, as indicated by the lack of interference of cyclosporin A on the appearance of tenofovir-equivalents in mesenteric plasma during a perfusion of rat ileum with tenofovir DF. A rapid intracellular conversion of tenofovir DF may impede efflux of the ester prodrug by P-gp-related efflux carriers, as the metabolites are not assumed to be a substrate of P-gp. This conversion may mask a possible P-gp-related efflux mechanism in rat ileum. Previous studies have indeed shown that the esterase-mediated conversion of tenofovir DF is much higher in homogenates from rat intestine compared with homogenates from Caco-2 monolayers (Van Gelder et al., 2000b). A faster metabolism resulting in a lower concentration of the “substrate” form may at least partially account for a lower effect of P-gp on the absorption of tenofovir DF in the in situ rat perfusion system.

In conclusion, our results illustrate that defined ester mixtures can be used to increase the intestinal absorption of tenofovir DF in an in vitro and an in situ absorption model and that the mechanism of action appears to be a complex interplay of different systems (metabolism and efflux). Our data also stress the importance of using different absorption models and the difficulty to make in vitro/in vivo correlations when using data obtained with one single absorption model; the differential expression of carriers and enzymes in different systems illustrates the difficulty of extrapolating observations between different systems/species. Further elucidation of the clinical relevance of the different biochemical absorption barriers (esterase activity, P-gp-related efflux) will be essential to optimize interference with this barrier as an absorption-enhancing strategy for ester prodrugs. Overall, these data confirm that formulation of ester prodrugs in combination with esters (as defined ester mixtures or as present in nature-
identical extracts) may be a simple and safe approach to regulate the intestinal absorption of ester prodrugs of acyclic nucleoside phosphonate analogs.

References


