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Species-dependent and site-specific intestinal metabolism of ester prodrugs

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Abstract

In order to select a species for drug absorption studies of ester prodrugs and to identify a possible absorption window with low esterase activity and hence increased absorption of the ester prodrug, the esterase activity was investigated in homogenates from various intestinal segments of different species. *p*-Nitrophenyl acetate and tenofovir disoproxil [bis(POC)-PMPA] were used as substrates for esterases. *p*-Nitrophenyl acetate is a model substrate for esterase activity, while tenofovir disoproxil (fumarate salt) is an ester prodrug of the potent antiviral nucleoside phosphonate analogue tenofovir. As esterase-mediated degradation during transepithelial transport may be a limiting factor for its oral absorption, targeting the prodrug to a region of the intestine with lower esterase activity may lead to an increase in oral absorption of the prodrug. The results obtained with *p*-nitrophenyl acetate and tenofovir disoproxil showed both a site-specific (duodenum $\geq$ jejunum > ileum $\geq$ colon) and species-dependent (rat > man > pig) degradation in intestinal homogenates. Degradation of tenofovir disoproxil in homogenates from Caco-2 monolayers (0.016 ± 0.003 nmol. s^{-1}. mg protein^{-1}) was low compared to its degradation in homogenates from human ileum (0.177 ± 0.052 nmol. s^{-1}. mg protein^{-1}). Rat ileum appears to be a suitable model to evaluate the influence of esterase activity on the oral absorption of the ester prodrug, as the degradation rate for tenofovir disoproxil (0.245 ± 0.054 nmol. s^{-1}. mg protein^{-1}) in rat ileum was similar to degradation in human ileum. The results also suggest that *colon targeting* may be a useful strategy to reduce the esterase-mediated degradation of ester prodrugs, hence resulting in a possible increase in their oral absorption.

Keywords: Esterase; Intestinal metabolism; *p*-Nitrophenyl acetate; Tenofovir disoproxil; Antiviral; Oral absorption

1. Introduction

The synthesis of ester prodrugs has been successfully employed in order to enhance the systemic delivery of pharmacologically active compounds

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with low oral absorption due to high hydrophilicity (Sinkula and Yalkowsky, 1975; Nielsen and Bundgaard, 1988). The more lipophilic nature of the ester prodrugs renders them more suitable for diffusion across the lipophilic membranes of the intestinal cells, thus improving intestinal absorption. The active compound is usually released by bioconversion when entering the systemic circulation or at the level of the target cells. However, there is increasing evidence from the Literature that degradation of a variety of drugs and prodrugs in the intestinal epithelium might limit their oral absorption. The effect of cytochrome P450 isoforms on intestinal absorption is well documented; it has been shown that CYP3A4 in intestinal mucosal cells is responsible for decreased oral absorption [e.g. cyclosporin A (Gan et al., 1996), fentanyl (Labroo et al., 1997)], for drug-drug interactions (Hebert et al., 1992) and for drug–food interactions (Chan et al., 1998).

Ester-type drugs are hydrolyzed by intestinal esterases, more specifically carboxylesterases (EC. 3.1.1.1.) (Inoue et al., 1979, 1980) belonging to the class of B-esterases, which are characterized by the fact that their enzymatic activity is inhibited by organophosphates (Walker and Mackness, 1983). Although various studies have described the role of esterases on intestinal absorption of ester prodrugs, further studies are necessary to clarify the possibility of interfering with esterase-mediated metabolism as a strategy to enhance the oral absorption of the ester prodrugs. In the present study, the esterase activity in homogenates from various intestinal segments of different species was investigated in order to select a species for drug absorption studies of ester prodrugs and to identify a possible absorption window with low esterase activity and hence increased ester prodrug absorption. The esterase-mediated degradation was investigated using *p*-nitrophenyl acetate and tenofovir disoproxil [bis(isopropyloxycarbonyloxymethyl)-(*R*)-9-(2-(phosphonomethoxy)propyl)-adenine, bis(POC)-PMPA]. *p*-Nitrophenyl acetate was used as a model substrate for esterase activity, while metabolism of tenofovir disoproxil was assessed in order to select a possible absorption window with a lower esterase-mediated degradation. Tenofovir disoproxil is a bis-ester prodrug of the potent antiviral nucleoside phosphonate analogue tenofovir (Tsai et al., 1995; Arimilli et al., 1997; Shaw et al., 1997; Suruga et al., 1998). Previous studies in which Caco-2 monolayers were used as an in vitro model to assess intestinal transport (Hidalgo et al., 1989), have demonstrated that the use of the ester prodrug enhanced the intestinal transport of tenofovir equivalents from 0.1% for tenofovir to 2.7% for tenofovir disoproxil (Naesens et al., 1998). Despite the increase in transepithelial transport, an important carboxylesterase-mediated hydrolysis of the bis-ester was observed, partially offsetting the efficiency of the ester prodrug to enhance the transepithelial transport of tenofovir. Metabolism and, consequently intracellular accumulation has previously been observed in the laboratory for bis(pivaloyloxymethyl)-9-[2-(phosphonomethoxy)-ethyl]-adenine [bis(POM)-PMEA, adefovir dipivoxil], a structural analogue of tenofovir disoproxil (Annaert et al., 1997).

2. Materials and methods

2.1. Materials

Tenofovir and tenofovir disoproxil (fumarate salt) were obtained through the courtesy of Dr N. Bischofberger from Gilead Sciences (Foster City, CA). Tetrabutylammonium hydrogen sulfate (Fluka, Switzerland), potassium dihydrogenphosphate (Merck, Darmstadt, Germany), methanol and acetonitrile (Biosolve, The Netherlands) were HPLC gradient grade. All chemicals used for culturing the cells were purchased from Gibco (Life Technologies, Merelbeke, Belgium). D-(+)-glucose, *p*-nitrophenyl acetate, *p*-nitrophenol, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Sodium potassium tartrate tetrahydrate and Folin-Ciocalteu's phenol reagent (Sigma), copper (II) sulfate pentahydrate (J.V. Baker B.V., Deventer, The Netherlands) and sodium carbonate (Acros Organics, Geel, Belgium) were used for protein determinations. Sodium fluorescein, sodium hydroxide and ammonia 25% solution were obtained from UCB (Leuven, Belgium), while DMSO was obtained

from Acros Organics (Geel, Belgium). Transport medium consisted of Hanks' balanced salt solution (HBSS) containing 25 mM glucose and 10 mM Hepes.

2.2. Caco-2 cell culture

Caco-2 cells were kindly provided by Dr Y. Schneider (UCL, Louvain-La-Neuve, Belgium). The cells were cultured as previously described (Augustijns et al., 1998). Briefly, 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 1–7. The Caco-2 cells were maintained in Dulbecco's minimum essential medium (DMEM) containing high glucose (4.5 g/l), glutaMAX™, 100 U/ml of penicillin, 100 mg/ml of streptomycin, 1% non essential amino acids and 10% fetal bovine serum and were grown in tissue culture flasks (75 cm^2, Nunc, Roskilde, Denmark). Cells were negative for Mycoplasma infection. For metabolism studies, cells of passage numbers between 74 and 88 (low passage numbers) and between 124 and 127 (high passage numbers) were used.

2.3. Preparation of intestinal homogenates from Caco-2 monolayers and from intestinal segments of rat, pig and man

Freshly scraped Caco-2 cells grown in a 75 cm^2 flask were homogenized in 5 ml transport medium using a Potter Elvejhem tube and pestle at 0°C. In case of rat and pig, duodenum, jejunum, ileum and colon were excised within 30 min after euthanizing the animals; subsequently, a segment ($\pm$ 5 cm) was cut along the longitudinal axis and washed with ice-cold transport medium to remove the intestinal contents. Human duodenum, ileum and colon were obtained from the Department of Abdominal Surgery, Catholic University Leuven (Belgium); the tissue was evaluated on anatomopathological bases to assure that it was non-pathological; the procedure was approved by the Committee of Medical Ethics and Clinical Research, University Hospitals Leuven (Belgium). The intestinal mucosa was removed by scraping the intestine from different species with a glass microscope slide. The scrapings were homogenized at 0°C in 5 ml of cold transport medium using a Potter Elvejhem tube and pestle.

After centrifugation of the crude Caco-2 and intestinal tissue homogenates at 10 000 $\times$ g for 10 min, the supernatants were harvested and kept at 0°C. Protein contents of all preparations were determined according to the method of Lowry et al. (1951) using BSA as a standard.

2.4. Degradation studies with p-nitrophenyl acetate in intestinal homogenates

After optimisation of the incubation conditions in preliminar experiments, *p*-nitrophenyl acetate was incubated with intestinal homogenates under linear conditions (time and protein contents). Protein contents for each segment of each species were adapted to balance between sensitivity of the analytical method and linearity of the esterase activity of the preparations from each species. A summary of the protein contents of the respective homogenates is reported in Table 1. The enzymatic reaction was started by spiking the intestinal homogenate with a prewarmed (37°C) solution of *p*-nitrophenyl acetate in transport

Table 1
Summary of the protein contents (mg protein ml incubation solution^{-1}) used during incubation studies with the model substrate *p*-nitrophenyl acetate (10–1000 μM) and (100 μM) tenofovir disoproxil

Species	Segment	*p*-Nitrophenyl acetate	Tenofovir disoproxil
Man	Duodenum	0.05	0.1
	Ileum	0.05	0.1
	Colon	0.05	0.2
Rat	Duodenum	0.025	0.05
	Jejunum	0.025	0.05
	Ileum	0.025	0.05
	Colon	0.05	0.1
Pig	Duodenum	0.05	0.5
	Jejunum	0.05	0.5
	Ileum	0.05	0.5
	Colon	0.05	1
Caco-1	/	0.044	0.5

medium (10–1000 μM) containing 1% of DMSO. The enzymatic degradation of *p*-nitrophenyl acetate to *p*-nitrophenol was followed spectrophotometrically (400 nm) over a time period of 2 mins; chemical degradation of *p*-nitrophenyl acetate could be neglected over this time interval. The initial velocity (nmol/s) of *p*-nitrophenol formation was calculated from the slope of the linear part of the absorbance versus time curve. Kinetic parameters (K_m/V_{max}) were calculated according to the Michaelis–Menten equation.

2.5. Degradation studies with tenofovir disoproxil in intestinal homogenates

Metabolism studies were performed under linear conditions at 37°C at a concentration of tenofovir disoproxil of 100 μM in intestinal homogenates at a specific protein content (Table 1). At predetermined time points, the reaction was stopped by adding 100 μl of the incubation solution to 900 μl of ice-cold methanol. After centrifugation at $10\,000 \times g$ (10 min, 4°C), 800 μl of the supernatant was evaporated by a gentle stream of air and redissolved in 800 μl of transport medium adjusted to pH 3.3 with HCl 0.05 N; 50 μl was injected into the HPLC system.

2.6. HPLC-analysis of tenofovir disoproxil and its metabolites

Tenofovir disoproxil and its metabolites tenofovir mono(ester) and tenofovir were analyzed using a high-performance liquid chromatographic system equipped with a model 600E Controller and Pump, a model 717plus autosampler and a model Lambda-Max UV detector at 260 nm (Waters, Milford, MA). UV signals were monitored and the obtained peaks integrated using a Digital personal computer running Waters Millenium 32 chromatography software. The column used was a homepacked polystyrene/divinylbenzene column (5×0.46 cm i.d., 8 μm particle size, 100 nm pore size; PLRPS, Polymer Labs, The Netherlands). The flow rate amounted to 1.2 ml/min. Mobile phase A consisted of a mixture of 980 ml buffer (10 mM potassium dihydrogenphosphate and 1 mM tetrabutylammoniumhydrogen sulfate adjusted to pH 8 with ammonia 25%) and 20 ml of acetonitrile. Mobile phase B consisted of 970 ml of the same aqueous buffer (adjusted to pH 5.5 with ammonia 2.5%) and 30 ml of acetonitrile. Mobile phase C consisted of 80% acetonitrile and 20% water. Separation was carried out with a gradient starting with 100% mobile phase A, changing to 91% of mobile phase B and 9% of mobile phase C after 4 min, followed by a linear gradient from 9 to 11% of mobile phase C over 5 min. After 9 min, the percentage of mobile phase C was increased to 23%, followed by a linear gradient to 30% mobile phase C over 7 min, and a return to the initial conditions over 1 min. In order to minimize chemical degradation of tenofovir disoproxil, samples were stored at 4°C. Concentrations of tenofovir disoproxil and its metabolites were determined using a calibration graph of each compound by means of standards. Tenofovir mono(ester) concentrations were determined using tenofovir disoproxil calibration curves, which was allowed since both esters had the same UV response at 260 nm.

2.7. Statistical analysis

Unless stated otherwise, experiments were performed in triplicate ($n = 3$). Results are expressed as average values $\pm$ S.D. One-way analysis of variance was performed to compare the site-specific degradation of tenofovir disoproxil in rat; pairwise comparison of all segments was done by Tukey's multiple comparison test.

3. Results and discussion

In a first set of experiments, the esterase activity in intestinal homogenates from several segments and several species was studied using *p*-nitrophenyl acetate, a model substrate for carboxylesterase activity. The kinetic parameters are represented in Table 2. In all species tested, segmental differences were observed in the degradation of *p*-nitrophenyl acetate, with the general order of decreasing activities: duodenum $\geq$ jejunum $>$ ileum $\geq$ colon. In Caco-2 homogenates, a slight increase in activity was observed in ho-

Table 2
Summary of the esterase-mediated degradation of *p*-nitrophenyl acetate (10–1000 μM) in homogenates from several segments of several species (mean ± S.D.; $n \geq 3$)

Species	Segment	V_{max} (nmol s^{-1} mg protein^{-1})	K_m (μM)
Rat	Duodenum	38.5 ± 10.6	353 ± 49
	Jejunum	28.6 ± 7.9	464 ± 89
	Ileum	16.1 ± 4.6	829 ± 300
	Colon	11.4 ± 4.2	2359 ± 1063
Pig	Duodenum	16.5 ± 10.6	339 ± 99
	Jejunum	12.9 ± 5.2	324 ± 70
	Ileum	4.4 ± 1.9	549 ± 152
	Colon	4.2 ± 0.9	490 ± 126
Man	Duodenum	14.5 ± 10.0	331 ± 48
	Ileum	10.0 ± 6.5	304 ± 114
	Colon	6.9 ± 1.5	511 ± 158

mogenates from higher passage numbers (6.5 ± 0.9 nmol. s^{-1}. mg protein^{-1}; passage numbers between 124 and 127) compared to lower passage numbers (4.1 ± 1.5 nmol. s^{-1}. mg protein^{-1}; passage numbers between 74 and 88), while the affinity of the esterases for *p*-nitrophenyl acetate remained the same (761 ± 299 μM for low passage numbers; 771 ± 287 μM for high passage numbers). This illustrates that the esterase activity did not change dramatically with increasing passage number of Caco-2 monolayers. Although the affinity for *p*-nitrophenyl acetate was much lower in human ileum and colon than in Caco-2 homogenates, the degradation rate of *p*-nitrophenyl acetate in Caco-2 homogenates was similar to that in homogenates from the lower part of the human intestine (Table 2).

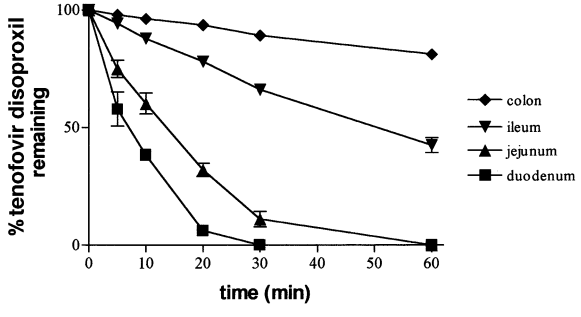

Fig. 1. Time-dependent degradation of tenofovir disoproxil (at an initial concentration of 100 μM) in homogenates from several segments of rat intestine (0.05 mg protein ml^{-1} in all cases), represented as percentage of intact bis-ester prodrug remaining.

The results obtained in different species confirm previously observed findings, that there is a significant species dependence (Yoshigae et al., 1998a) and site specificity (Narawane et al., 1993) in intestinal esterase activity towards *p*-nitrophenyl acetate. The highest activity was always observed in rat (as compared to pig or man) intestinal homogenates, and was higher for duodenum than for jejunum, ileum or colon homogenates.

In a second set of experiments, the intestinal degradation of tenofovir disoproxil was assessed. The prodrug is rapidly hydrolyzed during transepithelial transport, causing a reduction in its efficiency to act as enhancer of the transepithelial transport of tenofovir. Previous studies performed in the laboratory have shown that it is possible to enhance the transepithelial transport of tenofovir disoproxil by inhibiting its esterase-mediated metabolism in the Caco-2 model (Van Gelder et al., 1999); this study has illustrated the importance of intestinal degradation of the prodrug as a barrier to its oral absorption. The time-dependent degradation of tenofovir disoproxil to its mono(ester)-equivalent in homogenates from several segments of rat intestine is represented in Fig. 1: a significant ($P < 0.001$) site-specific degradation was observed at all time points. Fig. 2 shows the degradation rates of tenofovir disoproxil (100 μM) in homogenates from various segments of the three investigated species. A site-specific degradation occurred in all species tested, the highest degradation being observed in the duodenum. The general order of activity was similar to the order observed for *p*-nitrophenyl acetate, i.e. duodenum ≥ jejunum > ileum ≥ colon. The degradation of tenofovir disoproxil was lower than the degradation of *p*-nitrophenyl acetate. However, when the site-specific degradation of tenofovir disoproxil (100 μM) was compared with the degradation of *p*-nitrophenyl acetate (100 μM), a good correlation was obtained for rat intestine ($r^2 = 0.9964$) and pig intestine ($r^2 = 0.9991$). These results show the usefulness of *p*-nitrophenyl acetate as a model substrate to evaluate the site-specific

degradation of ester compounds in intestinal homogenates. In human intestine, however, the correlation between the degradation of tenofovir disoproxil and *p*-nitrophenyl acetate was much lower ($r^2 = 0.7369$).

In addition to a site specificity in esterase activity, species differences were also observed, the general order of decreasing activity being: rat > man > pig. The degradation of tenofovir disoproxil in human intestinal homogenates was relatively high, which is in concordance with studies performed on propranolol prodrugs (Yoshigae et al., 1998b). The degradation rate of tenofovir disoproxil (100 μM) in homogenates from Caco-2 monolayers amounted to 0.016 ± 0.003 nmol s^{-1} mg $protein^{-1}$, which is comparable to the degradation rate in homogenates from the lower part of pig intestine (colon $= 0.015 \pm 0.007$ nmol s^{-1} mg $protein^{-1}$). However, the esterase-mediated degradation of tenofovir disoproxil in Caco-2 homogenates was almost 7 times lower than in human colon, which is in contrast with a comparable degradation rate of *p*-nitrophenyl acetate in human colon homogenates and Caco-2 homogenates. These observations indicate that, although large similarities exist in the degradation profiles of *p*-nitrophenyl acetate and tenofovir disoproxil, the degradation of a prodrug ester cannot invariably be predicted using a model compound of esterase activity.

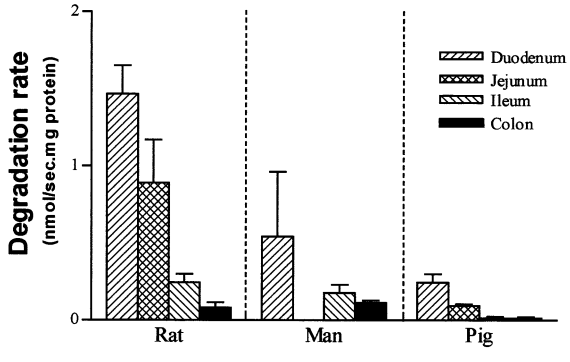

Fig. 2. Degradation rates of tenofovir disoproxil (at an initial concentration of 100 μM) in intestinal homogenates from various species and different segments. For rat and pig, $n = 3$; for human duodenum and ileum, $n = 5$; for human colon, $n = 7$; degradation was not determined in human jejunum. Values are corrected for chemical degradation.

The results suggest that rat ileum might be a suitable model to evaluate the influence of esterase activity on the oral absorption of tenofovir disoproxil, as the degradation of the ester prodrug in homogenates from this intestinal segment (0.25 ± 0.05 nmol s^{-1} mg $protein^{-1}$) is comparable to that of homogenates from human ileum (0.18 ± 0.05 mol s^{-1} mg $protein^{-1}$). This is somewhat contradictory to a previous statement that the rat is not a good model to test the oral absorption of ester prodrugs (Augustijns et al., 1998); this conclusion was based on the fact that the esterase activity in rat intestine is much higher than in pig intestine and on data from the Literature that pig intestine may be considered to be anatonomically and physiologically similar to human intestine (Kararli, 1995). However, as can be concluded from the results of this study, this comparison between pig and human intestine does not hold for intestinal esterase activity.

The relatively low esterase activity in Caco-2 cells might be responsible for an overestimation on transport enhancement by the ester prodrug in the Caco-2 model as compared to the in vivo situation. Yoshigae et al. (1998b) did not observe an important increase in the disposition of oral propranolol after administration of the compound as a prodrug, mainly due to an unsaturation of its metabolism during intestinal absorption in rat. They also reported a high hydrolase activity in human intestinal microsomes. The results further substantiate earlier observations concerning the importance of assessing the enzymatic activity in homogenates from the human intestine before extrapolating results from the Caco-2 system to humans (Augustijns et al., 1998). Other absorption models (for instance, ex vivo Ussing Chambers, in situ perfusion model) should be used in order to check the validity of results obtained with the Caco-2 model with respect to transport characteristics of ester prodrugs.

In conclusion, the data clearly demonstrate a species-dependent and site-specific degradation of the bis-ester prodrug tenofovir disoproxil in the order duodenum ≥ jejunum > ileum ≥ colon and rat > man > pig. These results confirm earlier findings with similar site-specific trends in rat (Augustijns et al., 1998) and rabbit (Narawane et

al., 1993) intestine. Furthermore, Ungell et al. (1998) observed an increase in permeability for hydrophobic compounds when descending the gastrointestinal tract; this might be due to the lipid composition of the cell membrane, resulting in different membrane fluidity. From the site-specific degradation findings, one may advocate *colon targeting* as a potential strategy to reduce esterase-mediated degradation of tenofovir disoproxil, and, possibly, to increase its oral absorption.

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