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In vitro behavior of a phosphate ester prodrug of amprenavir in human intestinal fluids and in the Caco-2 system: Illustration of intraluminal supersaturation

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Abstract

As a result of their improved aqueous solubility, the development of phosphate ester prodrugs is an interesting approach to increase intestinal absorption of poorly water-soluble drugs. Absorption of a drug from its phosphate ester prodrug is based on intestinal dephosphorylation of the prodrug which may result in intraluminal supersaturation of the parent drug, followed by an increased absorptive flux across the intestinal mucosa. In this study, we evaluated the behavior of fosamprenavir, a phosphate ester prodrug of amprenavir, in the Caco-2 system and in aspirated human intestinal fluids (HIF), both showing phosphatase activity. Starting from a solution of fosamprenavir in HIF, a supersaturated solution of amprenavir was generated and maintained during a time period sufficient for absorption. Moreover, supersaturation of amprenavir resulted in an enhanced flux across Caco-2 monolayers. To our knowledge, this is the first illustration of supersaturation in real intestinal media. Next, we showed an inhibitory effect of inorganic phosphate on the dephosphorylation of fosamprenavir, both in the Caco-2 model and in HIF. As a consequence, phosphate-buffered media, including fasted state simulated intestinal fluid (FaSSIF), are incompatible with the study of phosphate ester prodrugs and should be replaced with media containing a biorelevant phosphate concentration (0.4–1 mM) and another buffering compound such as 2-morpholinoethanesulfonic acid (MES).

Keywords: Amprenavir; Phosphate ester prodrug; Intestinal alkaline phosphatase; Caco-2; Human intestinal fluid; Intraluminal supersaturation

1. Introduction

As an alternative to formulation-related approaches, synthesis of water-soluble prodrugs is one of the strategies to increase the intestinal absorption of poorly water-soluble drugs (Fleisher et al., 1996). As a result of their relatively straightforward synthesis, inherent chemical stability and potentially improved dissolution/solubility characteristics, phosphate esters may be interesting oral prodrugs of poorly soluble drugs, especially for moderate to high permeability compounds (Heimbach et al., 2003a). Commercialized examples include estramustine phosphate, prednisolone phosphate, fludarabine phosphate, and fosamprenavir. Fosamprenavir is a phosphate ester prodrug of the poorly water-soluble HIV protease inhibitor amprenavir (0.036 mg/ml at pH 7 (Yu et al., 1999)). The calcium salt of fosamprenavir has been commercialized as Telzir® (Lexiva®), which should replace the standard amprenavir formulation (Agenerase®). With the Agenerase formulation, the intraluminal concentration of amprenavir is increased by the solubilizing excipient d-α-tocopheryl polyethyleneglycol 1000 succinate (TPGS) (Yu et al., 1999; Brouwers et al., 2006). Due to high amounts of TPGS required, this formulation suffers from a high pill burden (8 capsules of 150 mg as a standard dose). On the contrary, because of the improved solubility of fosamprenavir (pH-dependent: from 0.3 mg/ml at pH 7 to 54 mg/ml at pH 3.3) (Furfine et al., 2004), only two tablets (equivalent to 2 × 700 mg amprenavir) of the prodrug formulation suffice to obtain similar plasma concentrations of amprenavir (Falcoz et al., 2002; Chapman et al., 2004; Wire et al., 2006).

As transepithelial transport of phosphate ester prodrugs is in general very limited, formation of the parent drug before

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or during intestinal uptake is a prerequisite for absorption. Dephosphorylation of an orally administered phosphate ester prodrug to its parent drug is mediated by intestinal alkaline phosphatase (EC 3.1.3.1), present in the intestinal lumen and in the apical membrane of enterocytes. The activity of alkaline phosphatase can be affected by various components (e.g. amino acids, metal ions and inorganic phosphate) (Fernley and Walker, 1967; Komoda et al., 1982; Tardivel et al., 1992). Alkaline phosphatase activity in the intestine will therefore depend on intraluminal conditions, especially considering the complex and variable (intra- and inter-subject) composition of intestinal fluids. This dependency potentially affects the intestinal absorption of drugs administered as a phosphate ester prodrug. For instance, inorganic phosphate, a reaction product of dephosphorylation, has been recognized as a competitive inhibitor of alkaline phosphatase (Fernley and Walker, 1967). The physiological impact of this inhibition on phosphatase activity in serum has been investigated (Coburn et al., 1998). However, the potential impact of the modulatory effect of inorganic phosphate on intestinal phosphatase activity, and thus on drug absorption, has, to the best of our knowledge, not been explored yet.

If fast intraluminal dephosphorylation occurs, the higher solubility of a phosphate ester prodrug may result in parent drug concentrations at the intestinal mucosa that exceed the saturation solubility (supersaturation), possibly creating an enhanced absorptive flux. Whether or not a phosphate ester prodrug really increases the absorption of its parent drug will partly depend on the time window during which this intraluminal supersaturation is maintained in relation to the permeability of the parent drug (Heimbach et al., 2003b). Despite its importance, studies on supersaturation in relevant intraluminal conditions are rather limited. Kostewicz et al. (2004) investigated supersaturation and precipitation of weak bases upon entry into the small intestine in simulated intestinal fluids, but to our knowledge, supersaturation has not been studied yet in real intestinal media.

In the present study, we characterized the behavior of the phosphate ester prodrug fosamprenavir applied in the Caco-2 model system and in aspirated human intestinal fluids (HIF). Human Caco-2 monolayers express intestinal alkaline phosphatase at their apical membrane and have previously been used to study the absorption of phosphate ester prodrugs (Heimbach et al., 2003a,b). The use of intestinal fluids allowed to explore the influence of real intraluminal conditions on the stability of fosamprenavir. For reasons mentioned above, particular attention was paid to (1) the potential for maintained supersaturation in intraluminal conditions and the resulting effects on transepithelial transport, (2) the impact of modulation of phosphatase activity by physiological inorganic phosphate concentrations and (3) the use of biorelevant conditions in intestinal absorption studies.

2. Materials and methods

2.1. Materials

Amprenavir and fosamprenavir calcium were kindly provided by GlaxoSmithKline (Middlesex, UK). Stock solutions were prepared in DMSO and diluted at least 100× before use in experiments. Acetonitrile and methanol were purchased from Fisher Scientific (Leicestershire, UK). BDH Laboratory Supplies (Poole, UK) provided NaH_2PO_4, KH_2PO_4, NaOH and NaCl. Ammonium molybdate tetrahydrate, sodium taurocholate, glucose and 2-morpholinoethanesulfonic acid (MES) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Ascorbic acid was obtained from Certa (Brain-l'Alleud, Belgium) and Phospholipon 90G (lecithin) from Nattermann Phospholipid Gmbh (Köln, Germany). UCB (Leuven, Belgium) supplied sodium fluorescein. Hanks' Balanced Salt Solution (HBSS), HEPES and all chemicals for cell culture were purchased from Cambrex Biosciences (Walkersville, MD, USA). Water was purified with a Maxima system (Elga Ltd., High Wycombe Bucks, UK).

Transport medium consisted of HBSS, supplemented with glucose (final concentration 25 mM) and buffered with MES (10 mM) or HEPES (10 mM), before adjusting the pH (MES: pH 5.0–6.5; HEPES: pH 7.0–8.0). Fasted State Simulated Intestinal Fluid (FaSSIF) contained 3 mM sodium taurocholate and 0.75 mM lecithin in phosphate-buffer (NaH_2PO_4 28.7 mM, NaCl 100 mM, pH 6.5).

2.2. Caco-2 experiments

Caco-2 cells were purchased from Cambrex Biosciences (Walkersville, MD, USA) and grown in 75 cm^2 culture flasks (Nunc, Roskilde, Denmark) at 37 °C in an atmosphere of 5% CO_2 and 90% relative humidity. Cell culture medium consisted of Dulbecco's Modified Eagle Medium supplemented with 100 IU/ml penicillin–100 μg/ml streptomycin, 1% non-essential amino acids solution and 10% fetal bovine serum. Cells were passaged every 3–4 days (at 70–80% confluence) at a split ratio of 1–7.

For stability and transport experiments, cells with passage number between 40 and 60 were plated at a density of 40,000 cells/cm^2 on Costar® Transwell membrane inserts (3 μm pore diameter, 12 mm diameter; Corning Inc., NY, USA). Confluence was reached within 3–4 days after seeding and the monolayers were used for experiments 17–18 days post-seeding. Volumes amounted to 0.5 ml at the apical side of the monolayer and 1.5 ml at the basolateral side. On the day of the experiment, cells were rinsed and pre-incubated (30 min) with transport medium, after which the transepithelial electrical resistance (TEER) was measured with an EndOhm Voltohmmeter (WPI, Aston, England), as a control of monolayer integrity. Only monolayers with initial TEER values higher than 200 Ω cm^2 were used. The experiment was initiated by adding the incubation medium, containing fosamprenavir or amprenavir, to the donor compartment. While the pH in the basolateral compartment always amounted to 7.4, the apical pH was set at 6.5, unless specified otherwise. Samples were taken from the donor and acceptor compartment in function of time during 60 min and analyzed for both fosamprenavir and amprenavir. After 60 min, TEER was measured again. All results presented in this study are derived from experiments in which the TEER at the end of the incubation (1 h) was higher than 80% of the initial TEER

value. Finally, the absorptive flux of the hydrophilic marker sodium fluorescein (1 mg/ml) was measured as an additional control of monolayer integrity. The amount of sodium fluorescein appearing in the basolateral compartment was measured by UV spectrophotometry (Beckman Coulter DU 800, Fullerton, CA) at 490 nm.

2.3. Sampling of HIF

HIF was aspirated from three healthy volunteers (one man, two women; aged between 20 and 30 years), who were in the fasted state for at least 12 h. The procedure followed the tenets of the Declaration of Helsinki and was approved by the Committee of Medical Ethics of the University Hospitals Leuven, Belgium (ML3101). All volunteers provided written informed consent to participate in this study.

One double-lumen polyvinyl catheter [Salem Sump Tube 14 Ch (external diameter 4.7 mm), Sherwood Medical, Petit Rechain, Belgium] was introduced via the mouth and positioned into the duodenum (D2/D3). The position of the tube was checked by means of fluoroscopy. HIF was aspirated in function of time during 2–3 h (approximately 4–10 ml during each period of 15 min). HIF samples were collected on ice and centrifuged at 4000 g and 4 °C for 20 min. After measuring the pH (Hamilton Slimtrode, Bonaduz, Switzerland), the supernatant was stored at −30 °C. In all HIF samples, the inorganic phosphate concentration was assessed; for the experiments with fosamprenavir, HIF samples were pooled per subject.

2.4. Analysis of inorganic phosphate in HIF

The determination of inorganic phosphate concentrations in HIF was based on the formation of a blue phosphate–molybdate complex. HIF sample (100 μl) was diluted in 900 μl HCl 1 M and centrifuged for 5 min at 14,000 × *g*. After addition of 150 μl of the supernatant to 1 ml of HCl 1 M, the complex-forming reaction was initiated by adding 150 μl ammoniummolybdate solution (2.5% in water) and 150 μl ascorbic acid solution (4% in water). After 90 min at room temperature, the absorbance was measured at 820 nm. The calibration curve, made with KH_2PO_4 in water, was linear over the concentration range of 0.2–10 mM. The assessment of intraday repeatability resulted in a relative standard deviation ($n = 5$) of 5.3% at 0.3 mM and 1.1% at 9.0 mM. Deviations from the theoretical concentration amounted to +7.5% at 0.3 mM and +1.8% at 9.0 mM.

2.5. Stability of fosamprenavir in HIF

In order to assess the conversion of fosamprenavir to amprenavir in HIF, the prodrug (concentration as specified) was incubated at 37 °C in aspirated HIF. In function of time, samples of the incubation mixture were diluted 20–100 times (depending on the initial concentration) in an icecold solution of KH_2PO_4 (25 mM, pH 6.5) and methanol (40:60, v/v), thereby arresting the conversion. When the initial fosamprenavir concentration was above 100 μM, samples of the incubation mixture were first filtered through Chromafil GF/PET-45/25 filters (pore size 0.45 μm; Machery-Nagel, Düren, Germany) before dilution, in order to remove any possible precipitate of amprenavir. Neither amprenavir nor fosamprenavir did adsorb to the filter material used (data not shown). After centrifugation of the diluted solution (5 min at 14,000 × *g*), 50 μl of the supernatant was injected in the HPLC system (see below).

2.6. Determination of amprenavir solubility in HIF

The solubility of amprenavir in aspirated HIF was determined by mixing an excess of amprenavir powder in 2 ml of HIF on a 3D Rocking Platform STR9 (Stuart Scientific, Staffordshire, UK) at 37 °C. After 24 h, samples were filtered using Chromafil GF/PET-45/25 filters. A volume of 10 μl was then diluted in 1 ml of a mixture of KH_2PO_4 (25 mM, pH 6.5) and methanol (40:60, v/v). After centrifugation (5 min at 14,000 × *g*), 50 μl of the supernatant was injected in the HPLC system (see below).

2.7. HPLC analysis of fosamprenavir and amprenavir

After sample preparation as described above, fosamprenavir and amprenavir concentrations were measured by reversed-phase HPLC and fluorescence detection. A volume of 50 μl was injected into a Waters HPLC system consisting of a 600E controller and pump, a 717plus autosampler and a Novapak C-18 column (Waters, Milford, MA). Fluorescence signals (excitation 268 nm, emission 347 nm) were detected on a Jasco FP-1520 fluorescence detector (Tokyo, Japan). The column was equilibrated with a mobile phase consisting of 25 mM KH_2PO_4-buffer (pH 6.5) and methanol (70:30, v/v). After injection, the concentration of methanol was increased up to 60% over 1 min. Retention times of fosamprenavir and amprenavir amounted to 7.9 and 11.5 min, respectively. After elution, the column was flushed with acetonitrile:water (80:20, v/v) during 3 min and re-equilibrated with mobile phase during 4 min. The flow was maintained at a rate of 1.3 ml/min.

Calibration curves of fosamprenavir and amprenavir were linear over the concentration range of 0.008–3 μM. Samples were all diluted to fit in this range. Precision and accuracy were assessed by analyzing standard samples ($n = 5$) at 0.008, 0.08, 0.8 and 3 μM. The determination of the intraday repeatability resulted in relative standard deviations below 5.6% at all concentrations, both for fosamprenavir and amprenavir. The relative error remained below ±2.5% for fosamprenavir (except for −9.2% at 0.008 μM) and below ±5.0% for amprenavir.

2.8. Data presentation and statistical analysis

All data are presented as mean ± S.D. Data sets were compared using an ANOVA test combined with Dunnett's multiple comparison. Differences were considered significant at $p < 0.05$. All tests were conducted in triplicate.

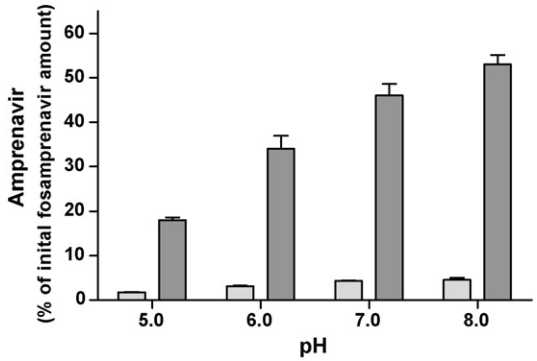

Fig. 1. Conversion of fosamprenavir to amprenavir in the Caco-2 system in function of apical pH. Fosamprenavir (10 μM) was incubated for 60 min in transport medium at the apical side of the monolayers. The amount of amprenavir found in the donor (dark grey) and acceptor compartment (light gray) is expressed as percentage of the initial amount of fosamprenavir (mean ± S.D., $n = 3$).

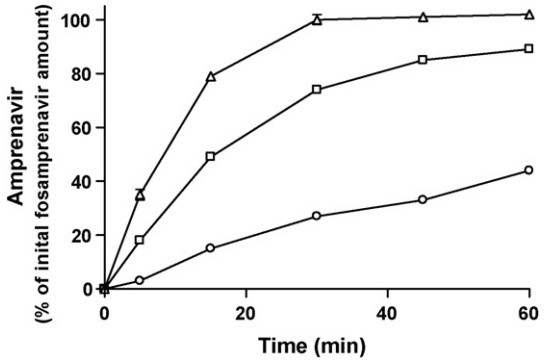

Fig. 2. Conversion of fosamprenavir to amprenavir in function of time in aspirated HIF from three subjects: A (□), B (◯) and C (△). The amount of amprenavir is expressed as percentage of the initial amount of fosamprenavir (mean ± S.D., $n = 3$).

3. Results and discussion

3.1. Fosamprenavir in the Caco-2 system

In a first set of experiments, the behavior of fosamprenavir was evaluated in the Caco-2 system. The stability of fosamprenavir in transport medium at 37 °C was confirmed in advance (data not shown). Upon incubation in the apical compartment of the Caco-2 system (60 min in transport medium pH 6.5), fosamprenavir was converted to amprenavir in a concentration-dependent way: 42 ± 4% at 10 μM, 33 ± 3% at 100 μM and 7 ± 1% at 1000 μM. The conversion increased with increasing apical pH, as illustrated in Fig. 1. While no intact prodrug could be detected in the basolateral compartment, a fraction of the total amount of amprenavir formed (about 8% at all pH values) was transported across the monolayer. When fosamprenavir was applied to the basolateral compartment, less than 4% was dephosphorylated to amprenavir and transport of neither amprenavir nor fosamprenavir to the apical compartment could be observed. These results are consistent with the necessity of prodrug degradation to parent drug by alkaline phosphatase present at the apical membrane of Caco-2 cells, prior to transepithelial transport. In vivo, transepithelial transport of intact fosamprenavir might be higher, as the paracellular route is more permeable in vivo compared to the Caco-2 model (Tavelin et al., 2003).

As amprenavir is a substrate for the efflux carrier P-glycoprotein (P-gp) (Yu et al., 1999; Brouwers et al., 2006) and inhibitors of P-gp (e.g. verapamil) have been reported to modulate hepatic alkaline phosphatase activity (Calhau et al., 2000), we co-incubated fosamprenavir with verapamil (100 μM) in the Caco-2 system. In the presence of verapamil, the total amount of amprenavir formed was not affected (44 ± 2% versus 42 ± 4%); however, the fraction transported increased from 10.1 ± 0.4 to 24 ± 1%. This indicates that verapamil only increases the permeability for amprenavir by inhibiting the P-gp functionality; it does not affect the intestinal dephosphorylation of fosamprenavir.

3.2. Fosamprenavir in HIF

HIF from three subjects (fasted state) was used for stability studies of fosamprenavir. The pH of the HIF samples from subjects A, B and C amounted to 6.8, 6.1 and 6.3, respectively. Upon incubation at 37 °C, fosamprenavir (10 μM) was converted to amprenavir (Fig. 2). This indicates the presence of phosphatase in HIF, originating from either pancreatic secretions or release by enterocytes. Therefore, HIF can be used as a physiologically relevant medium to study the degradation of phosphate ester prodrugs. As expected, the conversion rate varied significantly in HIF collected from different volunteers, probably as a result of variations in enzyme concentration and intraluminal conditions that may affect phosphatase activity (e.g. pH and inorganic phosphate concentration).

A comparison of the expression levels of different enzymes and transporters revealed a higher expression level of alkaline phosphatase in human duodenum compared to Caco-2 cells (Sun et al., 2002). Therefore, the conversion rate of phosphate ester prodrugs in vivo presumably exceeds the conversion rate observed in the in vitro models used in this study, which should be taken into account in the interpretation of the results.

3.3. Supersaturation of amprenavir

As mentioned in the introduction, the extent and duration of intraluminal supersaturation is important as it may result in an enhanced flux of the compound of interest; however, intraluminal supersaturation is a rather unexplored research area. The higher solubility of fosamprenavir compared to amprenavir, combined with intestinal phosphatase activity, may create local supersaturation of amprenavir. To confirm this, we determined the saturation solubility of amprenavir in HIF and compared this with the amprenavir concentration generated from a high amount of fosamprenavir in HIF. Due to the limited availability of aspirated HIF, these experiments were only performed in HIF from subject C (pH 6.3). No shift in pH was observed during the experiments. The equilibrium solubility of amprenavir in HIF, determined at 37 °C, amounted to 160.1 ± 0.5 μM, which

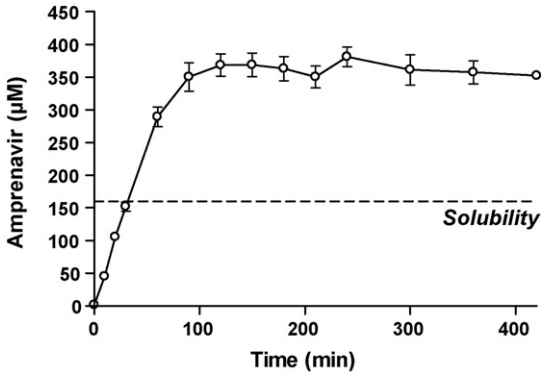

Fig. 3. Concentration of amprenavir (mean ± S.D., $n = 3$) in HIF in function of time upon incubation with fosamprenavir (500 μM) at 37 °C. Samples were filtered before analysis. After 60 min, the amprenavir concentration significantly ($p < 0.05$) exceeds the equilibrium solubility of amprenavir in HIF at 37 °C, represented by the dashed line.

is slightly higher than the solubility of amprenavir in transport medium (MES-buffered HBSS) at pH 6.3 (145 ± 3 μM). Starting from a solution of 500 μM of fosamprenavir in HIF, the amprenavir concentration was measured after filtration and is reported in Fig. 3. A concentration near 400 μM was reached in about 90 min, clearly exceeding the equilibrium solubility of amprenavir. Moreover, the amprenavir concentration remained at the same level for at least 7 h and no precipitation of amprenavir could be observed. This suggests the potential to create and maintain supersaturated intraluminal conditions for amprenavir during a time window sufficient for intestinal absorption.

Subsequently, the impact of supersaturation on the intestinal flux of amprenavir was investigated in the Caco-2 system. For this purpose, low (10 μM) and high (1 mM) amounts of fosamprenavir or amprenavir were dissolved or suspended in HIF and applied at the apical side of Caco-2 monolayers. As the use of HIF as apical medium can compromise monolayer integrity, transport was limited to 60 min. The cumulative amount of amprenavir detected in the receiver compartment in function of time is shown in Fig. 4. At a concentration at which both fosamprenavir and amprenavir completely dissolve in HIF (10 μM), the extent of transport is significantly lower when using fosamprenavir compared to amprenavir (Fig. 4A). This is due to the necessity of dephosphorylation of fosamprenavir before transepithelial transport; indeed, the amprenavir flux (based on the slope of the curve) increases in function of time. As dephosphorylation is expected to occur faster in vivo (see above), this difference may be clinically irrelevant. At a concentration of 1 mM (Fig. 4B), fosamprenavir completely dissolves in HIF, while amprenavir forms a suspension (concentration in solution: 150 μM). Upon 60 min incubation of these donor media in the Caco-2 system, transport from the fosamprenavir solution is slightly higher than from the amprenavir suspension, despite the initial need for dephosphorylation to generate a supersaturated solution of amprenavir. When a supersaturated solution of amprenavir (742 μM), generated in advance by incubation of fosamprenavir (1 mM) in HIF at 37 °C, was applied in the Caco-2 model, amprenavir transport clearly exceeded transport starting from the amprenavir suspension. In addition to enhanced passive diffusion due to an increased donor concentration, saturation of the efflux carrier P-gp may partly explain the increased flux from a supersaturated solution of amprenavir. However, the effect of P-gp on amprenavir transport is significantly decreased in the presence of HIF (Brouwers et al., 2006).

These results, obtained in real intestinal media, illustrate that intestinal dephosphorylation of fosamprenavir may result in a supersaturated solution of amprenavir, which in turn can lead to an increased flux across the intestinal mucosa.

3.4. Inhibition by inorganic phosphate

An essential step for intestinal absorption after administration of a phosphate ester prodrug is the dephosphorylation of the prodrug to its parent drug by intestinal alkaline phosphatase. Inorganic phosphate is a competitive inhibitor of alkaline phosphatase, but the impact of this inhibition on the intestinal processing of phosphate ester prodrugs has never been investigated. Therefore, we evaluated the effect of inorganic phosphate on conversion of fosamprenavir to amprenavir in the Caco-2 system and in HIF.

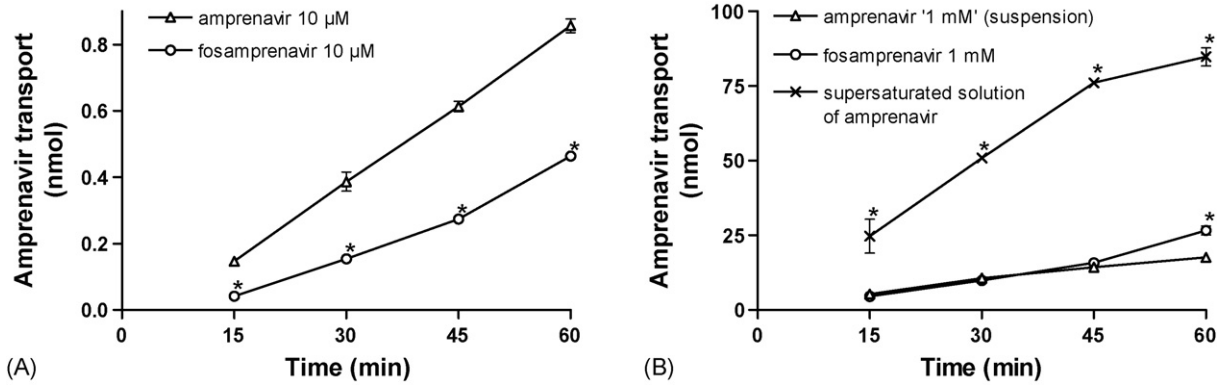

Fig. 4. (A and B) Amprenavir transport across Caco-2 monolayers in function of time starting from different apical conditions. All apical media were made in HIF from subject C. The initial conditions are indicated on the graph. The supersaturated amprenavir solution was generated by incubating fosamprenavir 1 mM in HIF at 37 °C prior to the Caco-2 study. Results are expressed as the cumulative amount of amprenavir transported to the basolateral compartment (mean ± S.D., $n = 3$). *Significantly different from transport from the amprenavir solution/suspension at the corresponding time point ($p < 0.05$).

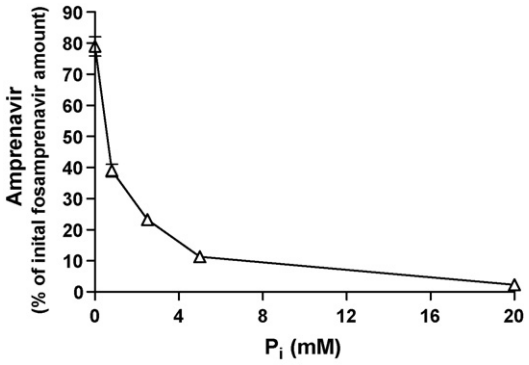

Fig. 5. The cumulative amount of amprenavir (in the donor and acceptor compartment) formed in the Caco-2 system upon apical incubation for 60 min with fosamprenavir (10 μM) in function of the inorganic phosphate concentration in the apical medium. Results are expressed as percentage of the initial amount of fosamprenavir (mean ± S.D., $n = 3$).

First, we studied the effect of an increasing inorganic phosphate concentration in the apical medium on the conversion of fosamprenavir (10 μM) in the Caco-2 model. For this purpose, transport medium was prepared without inorganic phosphate, whereupon different amounts of a stock solution of KH_2PO_4 were added, resulting in a final apical phosphate concentration from 0 to 20 mM. As depicted in Fig. 5, an increasing inorganic phosphate concentration resulted in a decreased conversion of fosamprenavir to amprenavir, confirming the inhibitory effect of inorganic phosphate on alkaline phosphatase. The permeability for amprenavir was not significantly affected by inorganic phosphate (data not shown). To evaluate the inhibitory effect of inorganic phosphate in real intraluminal conditions and at higher fosamprenavir concentrations, 500 μM of fosamprenavir was incubated in an HIF sample to which different amounts of a KH_2PO_4-stock solution were added, resulting in a final phosphate concentration of 0.4, 1.2 and 4.0 mM. Similar to the Caco-2 results, the conversion rate of fosamprenavir in HIF decreased with an increasing concentration of inorganic phosphate (Fig. 6).

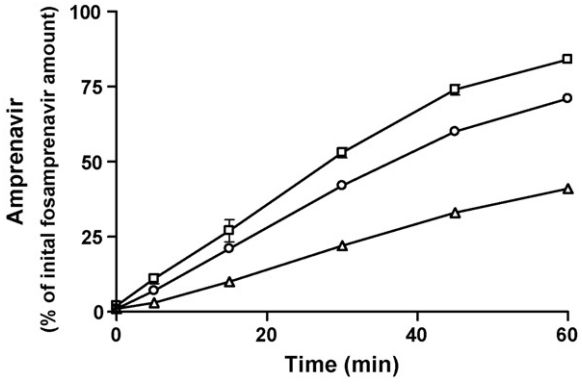

Fig. 6. Conversion of fosamprenavir (initial concentration 500 μM) to amprenavir in HIF containing different concentrations of inorganic phosphate. Different amounts of KH_2PO_4 were added to a pooled HIF-sample of subject C, resulting in a final inorganic phosphate concentration of 0.4 mM (□), 1.2 mM (◯) and 4.0 mM (△). The amount of amprenavir is expressed as the percentage of the initial fosamprenavir amount (mean ± S.D., $n = 3$).

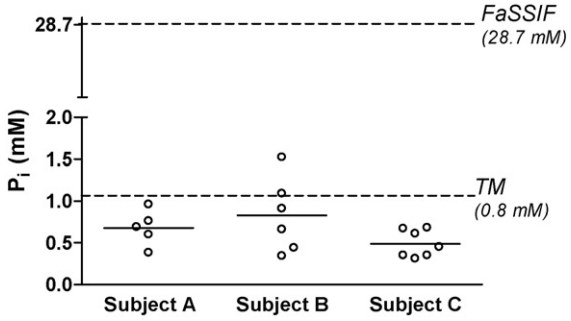

Fig. 7. The concentration of inorganic phosphate in HIF aspirates sampled from three subjects at different time points (sampling period: 2–3 h). The mean concentration is indicated for each subject by means of a full line. The dashed lines indicate the inorganic phosphate concentration in MES-buffered transport medium (TM) and in FaSSIF.

These results suggest the importance of the intraluminal inorganic phosphate concentration for the intestinal stability of phosphate ester prodrugs. Therefore, we determined the inorganic phosphate concentration in different HIF aspirates from three volunteers in the fasted state, sampled during a period of 2–3 h. Results are reported in Fig. 7. Taking into account the relatively low levels of inorganic phosphate (<1.6 mM) and the limited variability, the physiological impact of the inhibitory effect of inorganic phosphate on alkaline phosphatase will probably be restricted in the fasted state.

3.5. Biorelevant media

In recent years, the use of biorelevant media during the assessment of preclinical absorption data for drugs (solubility, dissolution, stability, permeability) is receiving growing attention. The composition of these media is based on real intestinal conditions (e.g. the presence of bile salts and phospholipids) and their use should increase the biorelevance of absorption studies, especially with respect to poorly water-soluble drugs. As the parent drug of a phosphate ester prodrug usually belongs to this category, the use of biorelevant media during in vitro absorption studies with phosphate ester prodrugs may be required. For instance, the creation and maintenance of supersaturated levels of the parent drug may be affected by the presence of surface-active agents. Based on the results of this study, however, some caution is required. Fasted State Simulated Intestinal Fluid (FaSSIF), often suggested to be relevant for the fasted state of the upper gastro-intestinal tract, is a phosphate-buffered medium that has previously been used in the Caco-2 system (Ingels et al., 2004). The concentration of inorganic phosphate in FaSSIF amounts to 28.7 mM, which is much higher compared to the phosphate concentration in aspirated HIF, as reported in Fig. 7. This should be considered when the process under study is affected by inorganic phosphate, as is the case for the intestinal processing of phosphate ester prodrugs. Therefore, we compared the use of FaSSIF versus the use of classic transport medium as the apical medium during incubation of Caco-2 monolayers with fosamprenavir. Table 1 shows that the conversion of fosamprenavir to amprenavir is more than 100-fold reduced in the presence of FaSSIF or blank FaSSIF (i.e. FaSSIF in the absence

Table 1
Amount of amprenavir in donor and acceptor compartments of the Caco-2 system after apical incubation for 60 min with fosamprenavir (10 μM) in different media

Medium	Amprenavir (% of initial fosamprenavir amount)	
	−Taurocholate, −lecithin	+Taurocholate 3 mM, +lecithin 0.75 mM
Transport medium (MES-HBSS)		
Donor	27.9 ± 1.7	37.1 ± 2.5[a]
Acceptor	1.9 ± 0.1	1.2 ± 0.1[a]
Blank FaSSIF (P_i-buffer 28.7 mM)		
Donor	0.2 ± 0.1[b]	0.37 ± 0.02[b]
Acceptor	n.d.	n.d.

Results are expressed as the percentage of the initial fosamprenavir amount (mean ± S.D., $n = 3$); n.d.: not detectable.
[a] Significantly different ($p < 0.05$) from the corresponding result obtained in the absence of taurocholate and lecithin.
[b] Significantly different ($p < 0.01$) from the corresponding result obtained in transport medium.

of taurocholate and lecithin). Transport of amprenavir could not be detected using (blank) FaSSIF. This indicates the incompatibility of FaSSIF, and more in general any phosphate-buffered medium, with intestinal absorption studies of phosphate ester prodrugs. In contrast to FaSSIF, classic transport medium (MES-buffered HBSS) contains inorganic phosphate at a concentration (0.8 mM) that falls within the range of intraluminal phosphate levels in the fasted state (Fig. 7). Therefore, an alternative biorelevant medium for use with phosphate ester prodrugs may consist of transport medium supplemented with taurocholate and lecithin. The use of this medium in the Caco-2 system resulted in a slightly increased extent of conversion of fosamprenavir to amprenavir compared to the use of standard (non-supplemented) transport medium (Table 1). However, the amount of amprenavir transported to the basolateral compartment decreased, probably due to micellar encapsulation of amprenavir.

4. Conclusion

As a phosphate ester prodrug as such cannot cross the intestinal epithelium, dephosphorylation to its parent drug by intestinal alkaline phosphatase is a necessary step in the absorption of the parent drug. Despite this importance, certain aspects of the dephosphorylation step are relatively unexplored, especially considering relevant intraluminal conditions. Therefore, we evaluated in this study the behavior of a phosphate ester prodrug of the poorly water-soluble amprenavir in the Caco-2 system and in HIF, both showing phosphatase activity. We particularly paid attention to (1) the generation of intraluminal supersaturation of amprenavir after dephosphorylation and (2) the potential impact of phosphatase inhibition by inorganic phosphate (Fig. 8).

The observation of a supersaturated amprenavir solution in HIF starting from fosamprenavir and resulting in an enhanced flux is, to our knowledge, the first illustration of the importance of supersaturation in real intestinal media. In vivo, the creation of local supersaturation of amprenavir after intake of a high dose of fosamprenavir, will most likely be essential for the generation of effective plasma concentrations.

The activity of intestinal alkaline phosphatase is clearly reduced in the presence of inorganic phosphate. As a consequence, the concentration of inorganic phosphate is an important variable that should be taken into account when using biorelevant conditions to study phosphate ester prodrugs. Therefore, phosphate-buffered media, including FaSSIF, should be replaced by media containing a biorelevant phosphate concentration (e.g. 0.8 mM in HBSS). The implications of the inhibitory effect of inorganic phosphate on intestinal phosphatase activity are probably limited in fasted conditions, regarding the relatively small variation in inorganic phosphate concentration in HIF from subjects in the fasted state. However, the intake of phosphate-rich food (e.g. milk, chocolate, soft drinks, ...) may increase the intraluminal phosphate concentration up to levels that can significantly affect the stability of phosphate ester prodrugs. This is under investigation in an ongoing study.

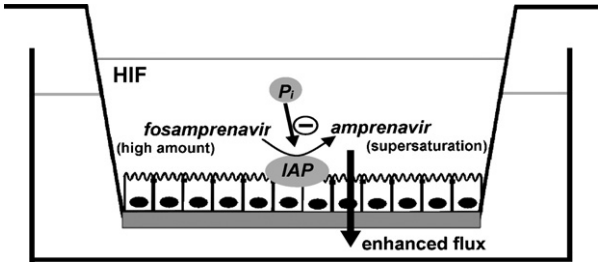

Fig. 8. Schematic overview of the behavior of fosamprenavir in HIF and in the Caco-2 system. The figure indicates the creation of supersaturated amprenavir concentrations, resulting in an enhanced flux of amprenavir, and the inhibition of intestinal alkaline phosphatase (IAP) by inorganic phosphate (P_i).

Finally, the results of this in vitro study warrant further studies, in which the in vivo intraluminal behavior of phosphate ester prodrugs is investigated and subsequently related to the pharmacokinetics of the parent drug.

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