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Research paper

Comparison of permeation enhancing strategies for an oral factor Xa inhibitor using the Caco-2 cell monolayer model

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Abstract

FXai, a direct inhibitor of the clotting factor Xa, provides high water solubility but poor membrane permeability due to multiple sites of ionization and a molecular weight exceeding 500 Da, making it a Class III drug according to the Biopharmaceutics Classification System. To overcome the ionization problem and increase the transcellular permeability, various ester and hydroxyamidine prodrugs exhibiting a reduced number of ionization sites were studied in the Caco-2 monolayer model for intestinal permeation. Alternatively, the potential transcellular permeation enhancement of Imwitor® 742 and the potential paracellular enhancement of three chitosan formulations were investigated in the same model. FXai has an apparent permeability (P_{app}) of about 1 nm/s, which is generally regarded as very low. The butylester-hydroxyamidine double-prodrug was found to provide a markedly increased permeability (40.4 nm/s) as did the co-application of chitosan (43.3 nm/s). Other prodrugs slightly increased permeability (1.3–9.2 nm/s) but were inferior to the previous attempts to enhance permeability while the Imwitor® admixture showed no effect (1.1 nm/s). Moreover, a bioactivating metabolism towards the hydroxyamidine mono-prodrug was detected in the Caco-2 cell permeation model. Although esterases were overexpressed and mainly located apically, an acceptable permeation was reached. In addition, the prodrugs triggered an efflux system that is not inhibited by verapamil but by quinidine, suggesting the involvement of an organic cation transporter.

Keywords: Factor Xa inhibitor; Caco-2 cell culture; Efflux transporter; Chitosan; Intestinal permeability; Prodrugs; BCS class III drug

1. Introduction

Delivering a drug via the oral route is preferred by patients for its convenience and is regarded as the route with the best compliance. Capsules and tablets can be produced cost-effectively and in large numbers. Therefore, oral bioavailability of a drug is an important property during lead optimisation in drug discovery. It depends on numerous factors, the most common being intestinal permeability, solubility during gastrointestinal transit, liberation from the dosage form, susceptibility to efflux and metabolism. The importance of especially solubility and intestinal permeability is reflected in the adoption of Amidon's Biopharmaceutics Classification System (BCS) [1] by the FDA in 2000, devised as a scientific basis to grant biowaivers for in vivo bioavailability and bioequivalence testing.

With the prerequisites for and mechanisms of gastrointestinal permeability being more researched and understood, beginning with Lipinski's Rule of Five [2], various in silico prediction models have come up as well as in vitro and ex vivo testing methods. The usage of these methods has provided some insight into the prerequisites of drug molecules to be successful oral candidates. Still, one is far

Abbreviations: P_{app}, apparent permeability; P-gp, P-glycoprotein; OCT, organic cation transporter; BCS, Biopharmaceutics Classification System; PSA, polar surface area; MCGs, medium chain glycerides; ABC, ATP-binding cassette; CTS, chitosan; TEER, transepithelial electrical resistance

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from predicting the permeability from structure with high precision. If sufficient permeability or solubility has not been realized during lead optimisation, prodrugs or specialized formulations can be used to cure the deficiencies of the drug. Prodrugs were first defined by Albert [3]. He described them as inactive compounds that undergo biotransformation before they exert a pharmacological effect. Another definition was given by Bundgaard [4] who describes them as pro-moieties attached to active moieties that can overcome the barriers that hinder the optimal use of the active principle. In this work, prodrugs are understood according to the Bundgaard definition.

FXai is a peptidomimetic drug that selectively inhibits factor Xa, a serine protease which is part of the prothrombinase complex that converts prothrombin to the clotting enzyme thrombin. The prothrombinase complex is located at the converging point of the intrinsic and extrinsic pathways of the coagulation cascade and poses an interesting target for anticoagulation therapy. Because of its peptide-like structure, FXai is highly hydrophilic. Under physiological conditions, it can be ionized in four positions: phenol, amidine, imidazoline, and carboxylic acid-function. A detailed list of possible ionic states across a pH range from 1 to 9 is given in Table 1, the corresponding structure of the uncharged molecule is depicted in Fig. 1. At pH 7, FXai is charged in four functional groups, making it an electrically neutral zwitterion. Its $\log P_{\text{octanol/H}_2\text{O}}$ in this state is −2.93, disclosing the compound as too hydrophilic to permeate across cellular membranes. In the upper small intestine, at pH 5–6, the phenolate will be protonated as well and $\log P_{\text{octanol/H}_2\text{O}}$ is at −3.32. FXai has a molecular weight of 526.5 Da. Paracellular passage of hydrophilic compounds like FXai across tight junctional gaps between epithelial cells is blocked for molecules with a molecular weight exceeding 300 Da in the small intestine and 200 Da in the large intestine and the thereof derived Caco-2 cells [5,6]. More precisely, it was found that cations of a molecular radius larger than 5 Å and neutral or anionic compounds larger than 3.5 Å do not pass the Caco-2 junctional gap [7,8], whereas in jejunal cells the pores can be 7–9 Å in size. Furthermore, the surface area of all tight junctions does only contribute 0.01% to the total intestinal surface area [9], making the transcellular route the more desirable pathway in general. Therefore, the physicochemical parameters are changed by prodrug formation.

To reduce the number of ionizable centers in the molecule, pro-moieties were attached to two functional groups. First, esterification of the carboxyl-group with either an ethyl- or butylgroup increased $\log P_{\text{octanol/H}_2\text{O}}$ to 1.9 and 2.1. Second, the additional hydroxylation of the aromatic amidine reduced lipophilicity to 0.3 and 1.4 (measured at pH 7.0) through its ability to form hydrogen-bonds, but it efficiently blocks protonization under weakly acidic conditions like the upper small intestine. A list of all prodrugs along with their physicochemical data is given in Fig. 1 and Table 2. $\log P$ values between 0 and 3 are regarded as an optimum range for orally active drugs and pose a good compromise between solubility and transcellular permeability [10,11], although lipophilicity represents only one aspect besides polar surface area (PSA), hydrogen bonds, molecular weight and other parameters. The pro-moieties will be detached from the parent once it has reached systemic circulation. The ester-bond will be easily cleaved to a large extent in intestinal and hepatic first-pass while the hydroxyamidine is supposed to be reduced in mitochondria from liver and other organs [12].

In contrast to a highly complex lead optimisation or prodrug synthesis, a specialized formulation design is often the method of choice to improve oral bioavailability. Amongst many other substances, medium chain glycerides (MCGs) have been discussed as transcellular permeation enhancers in many publications [13,14]. Most of them do not refer to MCGs in general, but more specifically to mixtures of mono-, di- and triglycerides of caprylic and capric acids, as they are sold by many different manufacturers. Because MCGs are a natural component of our daily food

Table 1
Effect of pH in 0.05 M buffered solutions on ionic state of dissolved FXai and its resulting $\log P$ and aqueous solubility

pH	Ionic states of FXai functional groups				$\log P$[b]	Aqueous solubility (g/L)
	Amidine pK_A[a] > 12	Imidazoline pK_A[a] 10.8	Phenol pK_A[a] 6.5	Carboxyl pK_A[a] 3.2		
1	+	+			−2.28	130
2	+	+			−2.33	24
3	+	+				
4	+	+		–		
5	+	+		–	−3.32	3
6	+	+		–		
7	+	+	–	–	−2.93	0.065
8	+	+	–	–		
9	+	+	–	–	−2.56	0.045

[a] The acid dissociation constants were determined with a pH-potentiometric method in 0.1 M hydrochloric acid at 25 °C. Approximately 3.8×10^{-4} M of drug was titrated in the presence of excess acid with 0.1 M potassium hydroxide.

[b] The partition equilibria between *n*-octanol and water, buffered at pH 1, 2, 5, 7, 9, were determined by the flask shaking method. Each test set consisted of three different volume ratios of the two phases, after equilibration they were separated by centrifugation (5000 rpm, 30 min) and subsequently analyzed by HPLC.

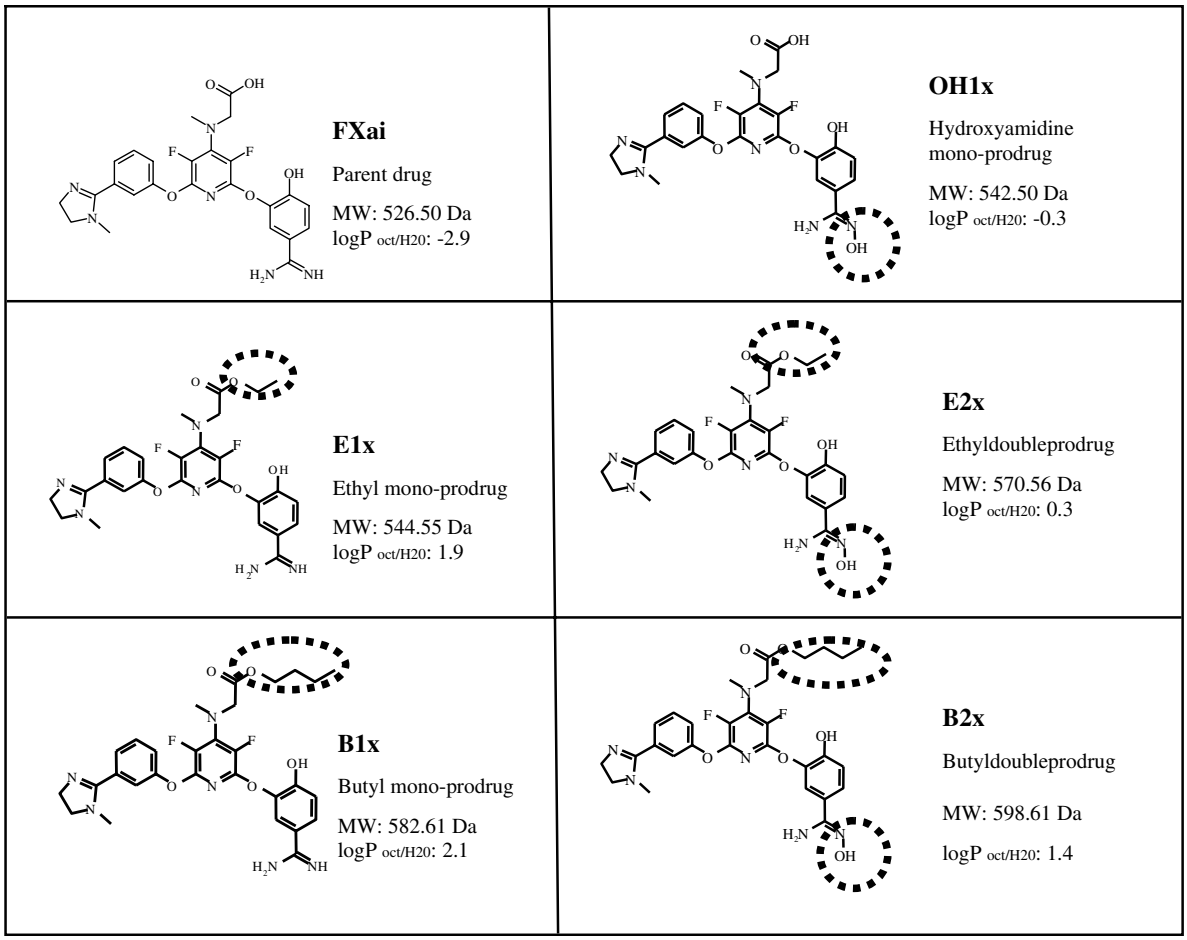

Fig. 1. Structures of FXai parent drug and its prodrugs along with their respective molecular weight and partition coefficient in octanol/water, the pro-moieties have been highlighted with a dotted line.

Table 2
Effect of prodrug formation on acid dissociation constants (pK_A) and partition coefficients ($\log P$)

Drug	Measured pK_A of the corresponding prodrugs				$\log P$[b]
	Amidine	Imidazoline	Phenol	Carboxyl	
FXai	>12	10.8	6.5	3.2	−2.9
OH1x[a]	4.9	12.0	7.9	3.6	−0.3
E1x[a]	>12	11.6	6.7	–	1.9
B1x[a]	>12	11.2	6.7	–	2.1
E2x[a]	4.8	11.2	7.9	–	0.3
B2x[a]	4.8	11.5	7.9	–	1.4

[a] Acronyms were formed according to the pro-moiety: B-butyl, E-ethyl, OH-hydroxyl, 1x-mono-prodrug, 2x-double-prodrug.
[b] $\log P$ was determined with the method described in Table 1 in 0.05 M PBS at pH 7.0.

intake, it is well known that they are perfectly tolerated upon oral administration, which might not be the case for other permeation enhancers.

The biodegradable polymer chitosan (CTS), a partially deacetylated form of chitin originating from crustaceans or fungi, has received much attention during the past decade for its ability to loosen epithelial tight junctions, thus allowing for larger molecules to permeate paracellularly into the interstitium [15] via the intestinal, nasal and pulmonary epithelia. Since this mechanism of action is limited to dissolved CTS, it can only take place in acidic environments where the amine-moieties will be protonated. To overcome this problem, more recent publications presented permanently quaternized ammonium moieties by alkylation with short-chain alkanes, thiols or carboxylic acids [16,17]. In general, the strategy to improve paracellular transport by tight junction opening has safety concerns, since bacteria and toxins from luminal chyme might as well reach systemic circulation. This is contrasted by the fact that CTS is taken as a dietetic treatment in amounts of up to 3.0 g/d and is licensed as a medicinal product in Europe [18,19].

Since the non-derivatized CTS is easily available and has a Ph.Eur. monograph, it was used in our experiments. FXai, being highly water soluble, has only poor chances of partitioning into the epithelial membrane. Therefore, an improvement of the paracellular permeation by loosening of tight junctional proteins is supposed to be more effective. The efficiency of CTS was reported to be dependent on polymer size and degree of deacetylation [20,21]. Because of this three different grades of CTS were used in the experiments.

2. Materials and methods

The permeation of FXai, its prodrugs and formulations was studied in the Caco-2 monolayer model of intestinal permeation. Caco-2 is a human adenocarcinoma cell line derived from colon. The experiments were carried out with Caco-2 tissue culture ACC 169, obtained from the DSMZ being the German Collection of Human and Animal Cell Cultures (Braunschweig, Germany) and which was used at passage numbers 42–57. Cells were seeded at a starting concentration of 100,000 cells/cm^2 and cultured for 21–28 days on Transwell No. 3460 bought from Corning Costar Co. (Cambridge, USA). The culture medium consisted of Dulbeccos' modified Eagle's medium, containing 3.7 g/L sodium hydrogen carbonate and 4.5 g/L D-glucose and was supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 1% NEAA (Non-essential amino acids), 2 mM glutamine and 10% fetal calf serum. All media components were purchased from Biochrom KG (Berlin, Germany). The medium was exchanged every second day. The cells were kept in an incubator at 37 °C and 90% relative humidity, supplemented with 5% CO_2. During the experiment a Hepes–carbonate buffer at pH 7.2 was used as transport medium.

Monolayer integrity was controlled by TEER measurements using a Millicell-ERS® ohmmeter from Millipore (Billerica, USA). Cell viability was monitored by a MTT-test utilizing the Biochrom-Alphakit from Biochrom (Berlin, Germany), which was adapted to match the well size as follows:

After end of the experiment, all wells were washed twice with PBS and culture medium was added, then the cells were incubated for 12 h. The medium was washed off twice and 250 μL of MTT-solution (5 mg/mL) was added followed by an incubation of two hours. The wells were washed twice and were left to dry for two hours. The blue formazan crystals were dissolved in 500 μL ethanol/hydrochloric acid 99/1, transferred to a 96-well plate and measured with a VIS-plate reader at 570 nm.

Apparent permeabilities (P_{app}) were calculated according to the following equation:

$$P_{app} = \frac{(\Delta Q/\Delta t)}{(60 * A * c_0)}. \quad (1)$$

where ΔQ is the cumulative amount [g], Δt is the sampling time [min], A is the transwell area [cm^2], c_0 is the starting concentration [g/cm^3].

The drug and prodrugs were manufactured by Schering AG Chemical Development (Berlin, Germany). Verapamil, probenecid and quinidine were purchased from Sigma Chemicals Co. (St. Louis, USA) and applied at a concentration of 20 μM. Three different grades of chitosan were bought from Sigma (Cat. Nos. #448869, #419419) and Fluka (Buchs, Switzerland) (#22741). Imwitor 742® was purchased from Synopharm (Barsbüttel, Germany). All other chemicals were of analytical grade.

Prodrug permeation experiments were performed as follows: 1.0 mg of the drug and prodrugs was dissolved in 250 mL transport buffer. 0.5 mL was applied apically. Samples were taken on the basolateral side after 15, 30, 60, 90 and 120 min by completely replacing the basolateral compartment with fresh transport buffer at each timepoint.

Distribution of metabolites in the apical and basolateral compartments was determined with E2x and B2x in concentrations of 5.0 mg/100 mL transport buffer. An apical to basolateral permeation experiment (A → B) and a basolateral to apical (B → A) permeation experiment were carried out. The A → B experiment was carried out as described above. In the B → A experiment, 1.5 mL of the test solution was placed basolaterally, 300 μL samples were drawn after 15, 30, 60, 90 and 120 min from the apical side. The apical compartment was replenished with 300 μL of fresh transport buffer after each timepoint to assure sink conditions.

To study the transcellular permeation enhancement of MCGs, 5.0 mg FXai was dissolved in 100 mL transport buffer. One hundred milligrams of Imwitor 742 (INCI: caprylic/capric glycerides) was added. The resulting coarse emulsion was homogenized with an Ultraturrax at 22.000 rpm for 5 min. 0.5 mL of the emulsion was applied apically. Samples were taken at the above-mentioned times.

In paracellular permeation enhancement studies 5 mg FXai was dissolved in 4 mL acetic acid (1% v/v), 40 mg of the respective chitosan was added and stirred until it had completely dissolved. Hepes–carbonate buffer (246 mL), adjusted to pH 6.0, 6.5 and 7.2, respectively, was added in the next step. Initially, some gel-like particles formed, so the resulting solution was kept stirring overnight. The pH was checked and adjusted again directly before the experiment.

Samples were analyzed using HPLC, a W717 autosampler together with a W2996 photodiode array detector and Millennium™ library search routines. The autosampler, detector and software were bought from Waters (Milford, USA). A Waters Spherisorb® ODS-2 column, 3 μm, 250 × 4.6 mm was used. The eluent consisted of water/acetonitrile 68:32 with 0.3% trifluoro acetic acid at a flow rate of 1.0 mL/min.

3. Results

3.1. Prodrug permeation enhancement

FXai, the hydroxyamidine, ethylester and butylester mono-prodrugs (OH1x, E1x, B1x), the ethylester-hydroxyamidine and butylester-hydroxyamidine double-prodrugs (E2x, B2x) were investigated in the Caco-2 model under identical experimental conditions.

It was found that in contrast to the parent FXai, mono- and double-prodrugs were able to penetrate the Caco-2 monolayer. But only small amounts of the prodrugs itself reached the interstitium. However, a larger quantity of an unknown metabolite was found. Using UV-spectral analy-

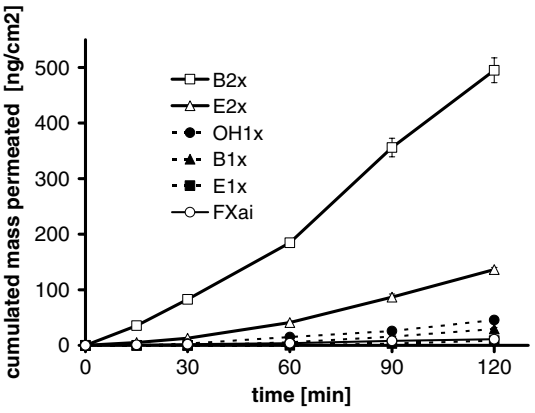

Fig. 2. Cumulative mass permeation of FXai ○ and its prodrugs B2x □, E2x △, OH1x ●, B1x ▲ and E1x ■ across the Caco-2 monolayer over time (mean sum of prodrug and corresponding ester cleaved metabolite ± SD, $n = 3$).

sis data generated from reference samples, the metabolite's UV spectrum was coinciding with OH1x in case of the double-prodrugs and with FXai for the ester mono-prodrugs, respectively.

In Fig. 2 the permeated mass sums of OH1x and the corresponding prodrug are depicted over the experiment time, Table 3 shows the corresponding P_{app} values. After addition of the two permeates, the recovery rate of each of the applied drug substances was in the range of 95–105%.

Interestingly, the metabolite was found in the apical and basolateral compartments at the end of the experiment. Concerning the double-prodrugs, more than 90% of their metabolite was found on the apical side (Table 4). When the double-prodrugs were placed on the basolateral side, an almost equal distribution of approximately 50% was reached on both sides after two hours and the total amount metabolized was slightly reduced (Table 5).

3.2. Prodrug efflux

Drugs transported by passive diffusion exhibit similar permeabilities both in the apical to basolateral direction and vice versa. If asymmetrical transport occurs, this is an indication of either active transport or metabolism involvement. To decide on that, the Efflux ratio (2) is calculated according to the following equation to predict the presence of active processes:

Table 4
Metabolite (OH1x) distribution after apical application of double-prodrugs after 120 min

Prodrug applied apically	Metabolite found apically		Metabolite found basolaterally	
	[ng/mL]	%	[ng/mL]	%
E2x	1325	92.8	103	7.2
B2x	3153	90.0	352	10.0

Intestinal ester cleavage was expected to occur intracellularly, leading to a 50/50 distribution of the metabolite by passive diffusion, since OH1x was found to be not actively transported. The uneven distribution suggests a stronger esterase activity in the apical membrane. This would represent a new absorptive barrier for the double-prodrugs as their metabolite OH1x is much less permeable. All experiments were done in triplicate.

Table 5
Metabolite (OH1x) distribution after basolateral application of double-prodrugs after 120 min

Prodrug applied basolaterally	Metabolite found basolaterally		Metabolite found apically	
	[ng/mL]	%	[ng/mL]	%
E2x	894	55.3	724	44.7
B2x	1110	43.4	1450	56.6

Even distribution of the metabolite in both compartments. In contrast to results in Table 3, basolateral application has circumvented the strong esterase activity in the apical region of the polarized cell, leading to the expected distribution of the metabolite.

$$R_{efflux} = \frac{P_{app}(B \rightarrow A)}{P_{app}(A \rightarrow B)}. \quad \text{where A is the apical compartment and B is the basolateral compartment} \qquad (2)$$

Taking into account the variation in biological models, an established "rule of thumb" predicts efflux if the Efflux ratio (R_{efflux}) exceeds factor 2 [22,23]. The two most promising prodrugs, E2x and B2x, were examined for their affinity to efflux transporters in a vice versa experiment, applying them to the basolateral side and taking samples from the apical side.

In contrast to the apical/basolateral experiment, the prodrugs crossed the cells in larger amounts while the metabolite OH1x played a minor role (Table 6). Calculating the Efflux ratio, one can see a strong efflux for both double-prodrugs (E2x: 57,4, B2x: 87,7), but not for their metabolite(OH1x: 0.6–0.7). In an attempt to characterize the efflux mechanisms involving verapamil as an inhibitor

Table 3
Apparent permeability of drug and prodrugs and their metabolite in apical to basolateral direction

Drug/prodrug	P_{app} [nm/s] after 120 min					
	FXai	OH1x	E1x	B1x	E2x	B2x
Itself	0.94 ± 0.12	2.74 ± 0.59	–	–	–	1.97 ± 0.74
Metabolite	–	–	1.25 ± 0.30	2.80 ± 0,40	9.2 ± 0.71	38.40 ± 2.12
Σ	0.94 ± 0.12	2.74 ± 0.59*	1.25 ± 0.30	2.80 ± 0.40*	9.21 ± 0.71*	40.37 ± 2.86*

Data represent means ± SD of three experimental determinations. * are significantly different ($p = 0.05$) from FXai value (unpaired Welch's t-test, assumed Gaussian distribution).

Table 6
Apparent permeability of FXai and its double-prodrugs in the basolateral to apical direction and the resulting efflux ratio

Drug/prodrug	Permeate	P_{app} [nm/s]	Drug/metab. relation [%]	R_{efflux}[a]
Fxai	FXai	2.1 ± 0.7	n.a.	2.2 ± 0.5
E2x	E2x	57.4 ± 2.1	81	>57.4
	Metabolite	13.5 ± 3.1	19	0.7
	Σ	70.9 ± 5.2	100	7.7 ± 0.2
B2x	B2x	87.7 ± 13.4	79	44.5
	Metabolite	23.7 ± 1.2	21	0.6
	Σ	111.4 ± 14.6	100	2.8 ± 0.2

[a] Calculated according to Eq. (2). For E2x, P_{app} (A → B) was set to 1 for calculation purposes.

of MDR1 (P-gp) [24] and, since the drugs are ionized, probenecid as an inhibitor of human organic anion transporters (hOATx) e.g. MRP1, MRP2 [25] and quinidine for human organic cation transporters (hOCTx) [26] were used, keeping in mind that all of them lack specificity for just one transporter family. As depicted in Fig. 3, apical to basolateral permeation of B2x was improved by neither verapamil nor probenecid but by quinidine (factor 2.3).

3.3. Transcellular permeation enhancement

Imwitor 742 was used as representative of other MCGs in the Caco-2 experiment to boost the transcellular permeability of FXai. The addition of Imwitor 742 did not result in an enhanced permeation, both mass flow and apparent permeability were approximately the same as without Imwitor (Table 7).

3.4. Paracellular permeation enhancement

Three different grades of CTS, varying in deacetylation degree (85%, 75%, 83.5%) and average molecular weight (low, high, medium), were investigated. All three chitosans improved the permeation of FXai at apical pH 6.0, P_{app} values ranging from 32 to 43 nm/s. However, no permeation was found at apical pH 7.2 (Table 8).

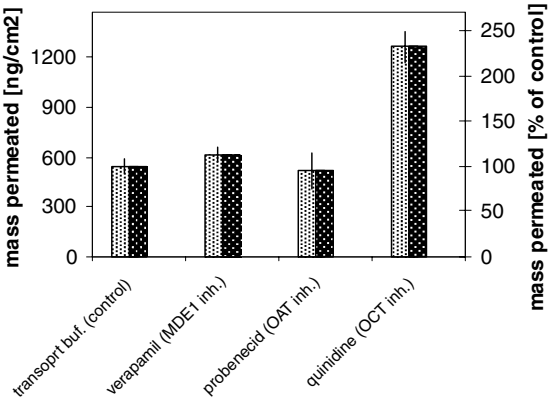

Fig. 3. Apical to basolateral mass permeation of B2x and its metabolite (6 μM) after 120 min ($n = 3$) without and with three different efflux inhibitors (20 μM each) in absolute numbers (light columns) and as percentage relative to transport buffer (dark columns).

Table 7
Effect of 0.1% Imwitor 742 on permeation of parent drug FXai across Caco-2 monolayers

Formulation	Transport buffer (control)	0.1 % Imwitor 742
P_{app} [nm/s]	0.94 ± 0.12	1.06 ± 0.51

Data represent means ± SD of three experimental determinations. * value is significantly different ($p = 0.05$) from FXai value (unpaired Welch's t-test, assumed Gaussian distribution).

A major TEER reduction of about 90% (Table 9) was observed at pH 6, at pH 7.2 it was significantly less pronounced (only about 10%). Furthermore, the observed TEER reduction was reversible to about 50% by subsequent washing with transport buffer. This was supported by results of a MTT-assay that showed a cellular survival of about 50% with only slight differences between the chitosan grades. TEER reduction, together with MTT- and washing step results, was taken as a sign for reduced electrical resistance by wider junctional gaps, so proving the functional efficacy of chitosans. However, chitosans in this experiment inherited some toxicity, since cellular death caused by all three grades was higher than in acidic buffer alone.

4. Discussion

4.1. Prodrug permeation enhancement

The results from Caco-2 cells are, to a certain scope, prone to culture conditions, passage number and seeding age and seeding accuracy. To be on the safe side, the resulting apparent permeabilities (P_{app}) are interpreted according to the following, arbitrarily chosen scheme: 0–20 nm/s was interpreted as poor permeation, 20–80 nm/s as moderate permeation and 80–∞ nm/s as good permeation.

The Caco-2 model is known to express many intestinal first pass enzymes, although their numbers, measured by their gene expression levels, do not correlate with the in vivo situation [27]. Of importance here are esterases, some of whom have been found to be more than 3-fold overexpressed in Caco-2, compared to human duodenum. On basis of this and the results of the spectral analysis, it was concluded that the unknown metabolite is the ester-cleaved OH1x or FXai in case of the ester mono-prodrugs. Since the cleavage is a prerequisite to form the active principle, adding the amounts permeated of prodrug and metabolite is allowed, coming to the following rank order of permeability: B2x > E2x > B1x > E1x > OH1x > FXai.

The most promising prodrug, B2x, possessed a 40-fold higher permeability than the parent drug, raising the active principle from poorly to moderately absorbed. The strong increase in permeability could neither be attributed to a single prodrug moiety nor is it the mere sum of both changes, since B1x and OH1x permeate only three times higher each. Taking into account that molecular weight,

Table 8
Comparison of FXai permeation across Caco-2 monolayers in combination with different types of CTS at acidic and slightly basic pHs

pH		P_{app} [nm/s]			
Apical	Basolateral	FXai (control)	FXai/# 448869 (85% deacetylated CTS)	FXai/# 419419 (75% deacetylated CTS)	FXai/# 22741 (83.5% deacetylated CTS)
6.0	7.2	0.76 ± 0.13	$32.48 \pm 1.75^*$	$39.70 \pm 0.63^*$	$43.32 \pm 2.84^*$
6.5	6.5	n.d.	n.d.	n.d.	34.88 ± 4.91
6.5	7.2	n.d.	n.d.	n.d.	39.66 ± 1.07
7.2	7.2	0.94 ± 0.12	0^*	0^*	0^*

Data represent means ± SD of three experimental determinations. * values are significantly different ($p = 0.05$) from corresponding FXai control (unpaired Welch's *t*-test, assumed Gaussian distribution). n.d., not determined.

Table 9
TEER reduction at acidic and slightly basic pH of different CTS grades compared to MTT test results and effect of washing step

	Buffer pH 6.0 (neg. control) (%)	# 448869 (%)	# 419419 (%)	# 22741 (%)
TEER reduction at pH 7.2	0	12.5	5.6	7.5
TEER reduction at pH 6.0	0	92.5	88.8	89.2
TEER reduction at pH 6.0 after additional washing	0	59.8	58.1	55.2
MTT cellular death at pH 6.0[a]	0	58.5	41.4	54.6

[a] Cellular death is accompanied by detachment of cells which leads to a proportional TEER reduction, therefore the comparison of both methods reveals the influence of CTS on tight junctions.

ionization state, log *P*, hydrogen bond donor and acceptor numbers have changed, it is reasonable that permeation properties completely differ from the parent compound.

If one looks at the metabolite distribution in the apical and basolateral compartments, one should expect an even 50/50 relation, because the double-prodrug would be taken up into the cell, be metabolized by cytosolic or mitochondrial esterases to OH1x, which would diffuse passively out of the cell in both directions. Because the concentration of OH1x in the apical well was much larger when the prodrugs were placed on the apical side, one can see an example of the polarized nature of mature Caco-2 cells, where esterase expression is much higher in the brush border membrane side. Once degraded to OH1x apically, permeation is distinctly lower than that of the double-prodrugs, thus explaining the abundance of OH1x in the apical compartment. Although Caco-2 seems to overexpress esterases, this issue has to be taken into account for in vivo applications as well. The fact that Prueksaritanont et al. [28] found esterase activities in the human small intestine which are comparable to human liver makes it clear that for ester prodrugs, these intestinal esterases may pose an additional barrier for systemic exposure if they already convert ester prodrugs to a large extent in the lumen. In case of the double-prodrugs, intracellular ester cleavage was pronounced enough to deliver a satisfactory amount of the bioactivated mono-prodrug to the basolateral compartment.

4.2. Prodrug efflux

FXai was a substrate to efflux mechanisms although its efflux ratio was not very pronounced. The double-prodrugs were very well able to penetrate the intestinal cells but had a high affinity to an efflux system and were rapidly transported out. If the ester-bond was cleaved intracellularly before, the metabolite was able to pass into the interstitium, since it did not show directional transport. If the efflux ratio is calculated only for the sum of prodrug and metabolite, it falsely underpredicts the efflux susceptibility of the prodrugs. Since an active secretory process seems to play an important role in transport of the double-prodrugs, its mechanism needs further investigation. Prodrug efflux under in vivo conditions is likely to become more intense because many ABC-transporters are present but underexpressed in Caco-2 culture. MDR1 and OAT transporters can be excluded since neither verapamil nor probenecid improved permeation in the basolateral direction. Instead, the transporter was affected by quinidine, which can lead to the conclusion that OCT isoforms 1–3 are involved in the efflux process since they are known to be inhibited by that drug. OCTs have been found in Caco-2 [27]. The symport has been described for hOCTN2, a carnitine reuptake transporter in kidney tissue [29]. Little is known on selective inhibition of specific isoforms, apart from Bourdet et al. [26], and on structure/affinity relationships between drugs and OCTs. Other than for MDR1, no pharmaceutical excipients have been reported so far that can interact with OCTs. In consequence, further prodrug optimisation as well as a specialized formulation design for the double-prodrugs appear to have little chance of success. Searching for a formulation that enhances the permeation of the parent FXai via the transcellular or paracellular route seems more promising.

4.3. Transcellular permeation enhancement

Whereas it is well known that MCGs are able to enhance the transcellular permeation of drugs [13,14], literature on their actual mechanism of intestinal permeation enhancement on a cellular level is scarce. An alteration of the intestinal cell membranes is generally assumed [30] in the case of anionic surfactants. It is still unclear whether it is a change in lipid order, orientation, fluidity or even a

solubilization of membrane lipids or proteins. Recently, a computational approach was made to simulate the interaction of drugs and small solutes with the lipid bilayer [31]. Functional cell membranes are fluid mosaics of proteins within a lipid bilayer matrix, their tails forming a hydrophobic core region with hydrophilic headgroups. In the computational simulation, successful permeates had the capability to interact with phospholipids via hydrogen bonds and hydrophobic interactions and they were sterically able to merge with the bilayer, thereby maximizing their interactions. It is imaginable that mono-and diglycerides might interact with the bilayer in the same way, thus disturbing its structure and allowing drugs to permeate. To explain the lacking effect of Imwitor 742, it can be guessed that FXai, although it can be twisted into many conformations, will only orientate to the hydrophilic headgoups since it is missing a fitting hydrophobic region. FXai is therefore penetrating only into the outer regions of the bilayer and cannot profit of the membrane alterations caused by Imwitor®.

4.4. Paracellular permeation enhancement

As already outlined in the introduction, CTS exerts its permeation enhancing effect on tight junctions only in an acidic environment because the polymer has to be positively charged. This is known in the literature and led to the synthesis of fixated cationic charges by quaternization. The effect was confirmed in our study by observing a strong TEER reduction, which was neither linked to cellular death determined with a MTT-assay nor was it irreversible since the additional washing step was able to rise the TEER value again. Permeation of FXai was raised 40-fold, which can be seen as moderate but sufficient at neutral pH, an unchanged permeability of about 1 nm/s was expected. Surprisingly, no permeation at all was found. Taken into consideration that CTS is also used as retarding agent, an additional experiment was set up to further investigate this. It was found that at pH 7.2, CTS formed stable gel-like particles with CTS # 22741 in a complex of a molar ratio of about 150:1.The particles were not visible to the eye, but could be separated by 0.4 μm filtration. The complex was stable against three washings with pH 7.2 buffer and no FXai was soaked out. The drug was completely recovered from the particles after they had been dissolved in 0.1 N hydrochloric acid. If there was any drug release from the invisible particles during the Caco-2 experiment, it was below the detection limit.

CTS is described to have no cytotoxic effects, so the mild toxicity found in these experiments should be attributed to the unrefined nature of the batches used.

The use of CTS proved to be a promising strategy within the boundaries of the in vitro model, but it will limit the absorption window in the gastrointestinal tract to the upper small intestine, where an acidic pH is prevailing. An optimisation through the use of quaternized chitosans is possible.

In conclusion, FXai turned out to be a difficult molecule to develop, its high hydrophilicity blocked transcellular absorption which could not be improved by using the pharmaceutical acceptable surfactant Imwitor® 742. Paracellular permeation enhancement in CTS-formulations, necessary due to FXai's high molecular weight, worked well in the Caco-2 model but was questionable for in vivo applications due to safety reasons that might come up during co-application of other drugs, especially if they have a small therapeutic range. Increasing the lipophilicity of the molecule through prodrug formation led to considerably improved permeation on the one hand. On the other hand, it became obvious that the prodrug approach might open up new questions like prodrug efflux and intestinal first-pass under in vivo conditions.

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