Department of Pharmaceutical Technology and Biopharmaceutics[1], Johannes Gutenberg University, Mainz, Germany; Absorption Systems LP[2], Exton, Pennsylvania, USA; Institute of Clinical and Functional Anatomy[3], University Medical Center, Johannes Gutenberg University, Mainz, Germany

Dissolution/permeation: The importance of the experimental setup for the prediction of formulation effects on fenofibrate *in vivo* performance

K. Forner[1], I. Hidalgo[2], J. Lin[2], M. Ackermann[3], P. Langguth[1,*]

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**Corresponding author: Prof. Dr. Peter Langguth, Department of Pharmaceutical Technology and Biopharmaceutics, Johannes-Gutenberg University, Staudingerweg 5, 55128 Mainz, Germany*
langguth@uni-mainz.de

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The evaluation of formulation strategies in early drug development requires *in vitro* methods that correctly predict oral drug absorption. The present study aimed to define key parameters for the predictability of dissolution/permeation experiments. Dissolution/permeation experiments were performed in various setups. The IDAS1 chamber, Ussing chamber, and transwells were used as Caco-2 cell based dissolution/permeation models to study the impact of chamber volumes and vertical or horizontal membrane orientation. Dissolution/permeation experiments in Ussing chambers with excised rat intestine were performed to depict the impact of the permeation membrane. Fenofibrate served as model compound in formulations of different particle size. Caco-2 cell based dissolution/permeation experiments with a vertical membrane orientation correctly depicted the formulation effect seen in vivo. The chamber volumes did not affect the outcome. A horizontal membrane orientation achieved no distinction. Experiments using excised rat intestinal sheets did not distinguish between the formulations, and the permeation was much lower than across Caco-2 cells. Mucus might present an artificially enhanced barrier for fenofibrate. Factors that greatly affected the predictivity of the dissolution/permeation experiments were thus the type and orientation of the permeation membrane, whereas chamber volumes only had a minor influence. Vertically mounted Caco-2 cells resulted in the best formulation distinction.

1. Introduction

Dissolution and permeability behaviour of a drug essentially determine its oral absorption. While both processes are often determined separately *in vitro*, they occur simultaneously *in vivo*. Therefore, a variety of experimental setups were developed that allow for dissolution and permeation to happen and be determined concurrently. To the best of our knowledge, despite the diversity of introduced setups, no comparison has been reported and the key factors for predictive results have not been determined.

One of the model compounds that stressed the importance of combined dissolution/permeation experiments is fenofibrate (Buch et al. 2009; Pestieau et al. 2016).

As a typical BCS class II drug, formulation strategies that ameliorate the dissolution behaviour greatly affect its absorption, and have hence been applied to improve its bioavailability (Guichard et al. 2008; Maciejewski and Hillemann 2008). In order to predict the impact of an improved dissolution behaviour on fenofibrate *in vivo* absorption, several studies showed that only the combination with a permeation step allowed for predictive dissolution experiments (Buch et al. 2009; Pestieau et al. 2016; Hens et al. 2015). These studies applied a variety of experimental setups. The aim of the present study was to define parameters that influence the predictive ability of dissolution/permeation experiments by comparing different experimental systems. The focus of this study was to determine the importance of the origin and orientation of the permeation membrane.

Dissolution/permeation experiments were performed with Caco-2 cells as permeation membrane in i) In-vitro Dissolution Absorption System 1 (IDAS1) chambers, a setup adapted from the dissolution/permeation system developed by Kataoka et al. (2003), ii) conventional Ussing chambers, a setup with different chamber volumes compared to IDAS1 chambers, and iii) transwells, where, as opposed to the other systems, the Caco-2 cell membrane is mounted horizontally rather than vertically. A schematic illustration of the different setups is shown in Fig. 1. To investigate the impact of the type of the permeation membrane and a possible role

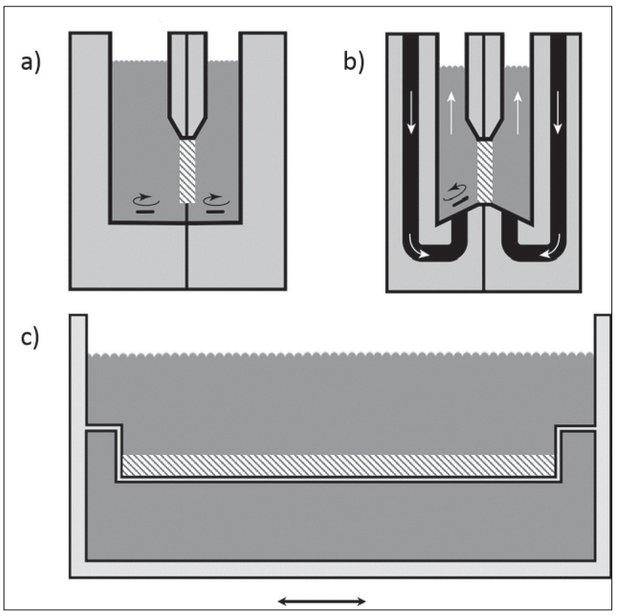

Fig. 1: Schematic illustration of the applied dissolution/permeation setups, a) dissolution/permeation in the Ussing Chamber setup, b) dissolution/permeation in the IDAS1 setup, c) dissolution/permeation in the transwell setup (white arrows indicate oxygenation with carbogen).

of the mucus barrier in *in vitro* drug permeation, excised rat intestinal mucosa was utilized in dissolution/permeation experiments in Ussing chambers.
The chosen formulations contained nanosized and micronized fenofibrate, with a higher *in vivo* absorption of the nanosized formulation (Maciejewski and Hillemann 2008).

2. Investigations and results

The simultaneous evaluation of dissolution and permeation across Caco-2 cells mounted in IDAS1 chambers (Fig. 2) and in Ussing chambers (Fig. 3) resulted in a linear increase of the permeated amount over time for both formulations. The permeated amount of fenofibrate from the nanosized formulation was significantly higher than that from the micronized formulation.

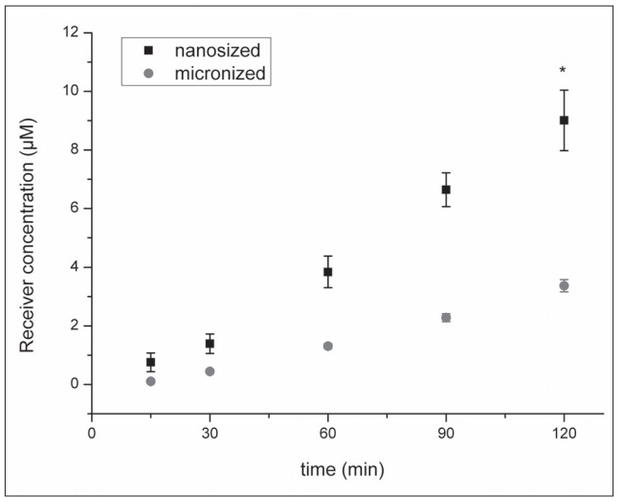

Fig. 2: Fenofibrate concentration in the receiver chamber of dissolution/permeation experiments across Caco-2 cell monolayers in IDAS1 chambers (mean ± SEM, n = 6).

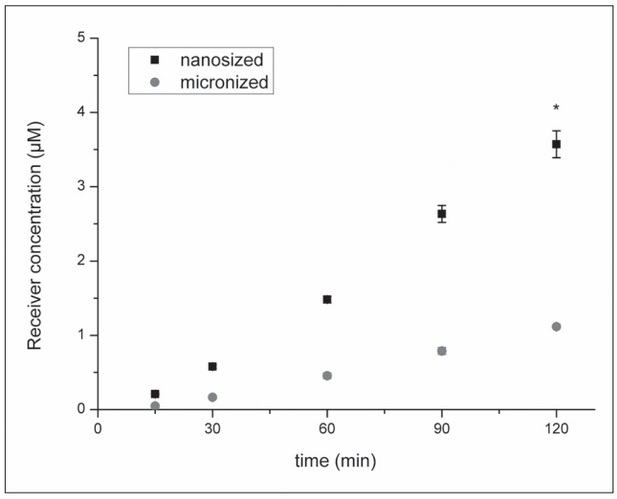

Fig. 3: Fenofibrate concentration in the receiver chamber of dissolution/permeation experiments across Caco-2 cell monolayers in Ussing chambers (mean ± SEM, n = 6, * indicates significant difference between the formulations).

The apparent permeability of atenolol was 0.19 x 10^{-6}cm/s and 0.12 x 10^{-6} cm/s for the nanosized and micronized formulation, respectively. This permeability was below the maximum value of integrity as defined above (0.5×10^{-6} cm/s), and was hence regarded as intact tissue.
Comparing the permeation of fenofibrate across Caco-2 monolayers in the IDAS1 setup with that in the Ussing chamber setup, the permeated amount was smaller in the latter, but the calculated fluxes were in the same range and showed a comparable ratio between the nanosized and micronized formulations (Table). For a relation to *in vivo* data, the ratio of both formulations (i.e. nanosized/micronized) was calculated as 1.7 for $AUC_{0-\infty}$ and 4.4 for C_{max}.

Table 1: Calculated fluxes (nmol/cm²/min) of the nanosized and micronized fenofibrate formulations in various dissolution/permeation setups

Membrane	Setup	Flux nanosized formulation	Flux micronized formulation	Ratio A/B
Caco-2	IDAS1	0.252	0.097	2.60
	Ussing chamber	0.147	0.046	3.18
	Transwell	0.258	0.267	0.97
Excised rat intestinal sheets	Ussing chamber	0.007	0.009	0.79

The simultaneous assessment of dissolution and permeation experiments across Caco-2 cells mounted horizontally (transwell) indicated a linear increase of the permeated amount over time (Fig. 4), with no differentiation between the formulations. The flux was higher than in the other systems (Table).

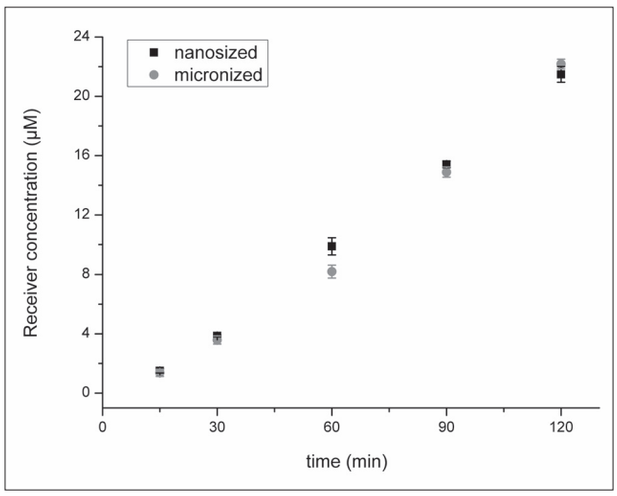

Fig. 4: Fenofibrate concentration in the receiver chamber of dissolution/permeation experiments across Caco-2 cell monolayers in transwells (mean ± SEM, n = 3).

The permeated amount of fenofibrate across excised rat intestinal mucosa was below the detection limit until the 90 min time point (Fig. 5). The permeated amount did not differ between the two formulations. It was significantly lower than for Caco-2 cells, with a much higher relative standard deviation. Calculated fluxes are shown in the Table.
With regard to fenofibrate metabolism (Fig. 6), the percentage of detected fenofibric acid out of the total permeated amount was above 70 % and relatively similar for all Caco-2 cell based experiments. It was much lower in experiments with excised rat intestinal mucosa (Fig. 7).

3. Discussion

Drug formulation development requires that decisions on the most promising strategy for an improved *in vivo* performance are made as early as possible. For the evaluation of BCS class II drugs, *in vitro* dissolution tests are widely performed and generally correlate well with the *in vivo* absorption (Galia et al. 1998; Wei and Löbenberg 2006). However, shortcomings of dissolution experiments

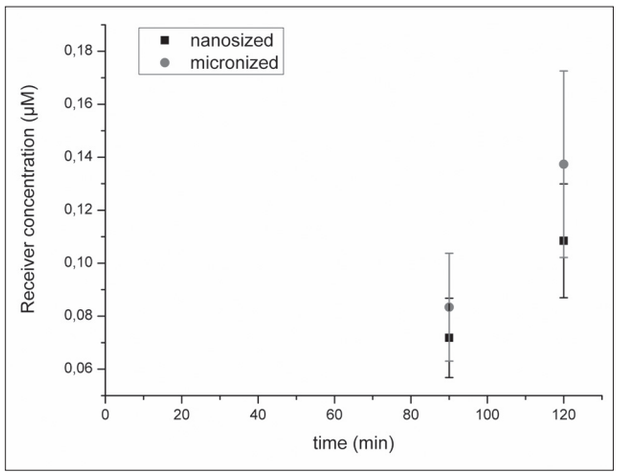

Fig. 5: Fenofibrate concentration in the receiver chamber of permeation and dissolution/permeation experiments across excised rat intestinal sheets in Ussing chambers (mean ± SEM, n = 6).

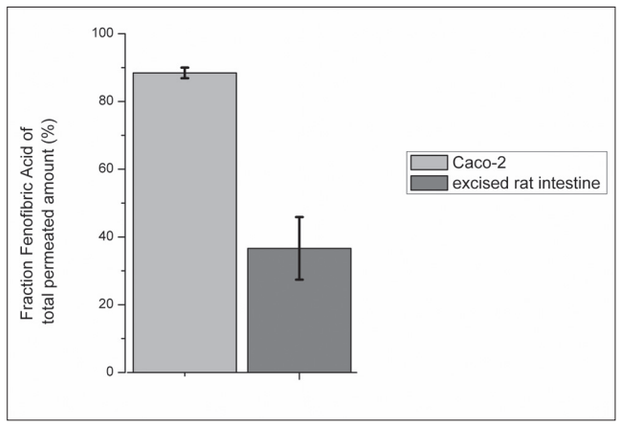

Fig. 7: Fraction of fenofibric acid out of the total amount (i.e. metabolised plus unmetabolised) of fenofibrate in the receiver chamber of permeation and dissolution/permeation experiments across Caco-2 cells and excised rat intestine.

have been reported for the investigation of formulation effects. Pestieau et al. (2016) reported that *in vitro* dissolution experiments were not able to depict the different performance of three fenofibrate formulations. Hens et al. (2015) reported the opposite for other fenofibrate formulations, where an increase in dissolution did not result in an increase in absorption, much like the negative solubility/permeability reported by Beig et al. (2015a; 2015b) for different BCS class II drug formulations. In these cases, *in vitro* models that accounted for both, dissolution and permeation, improved the predictability of the experiments.

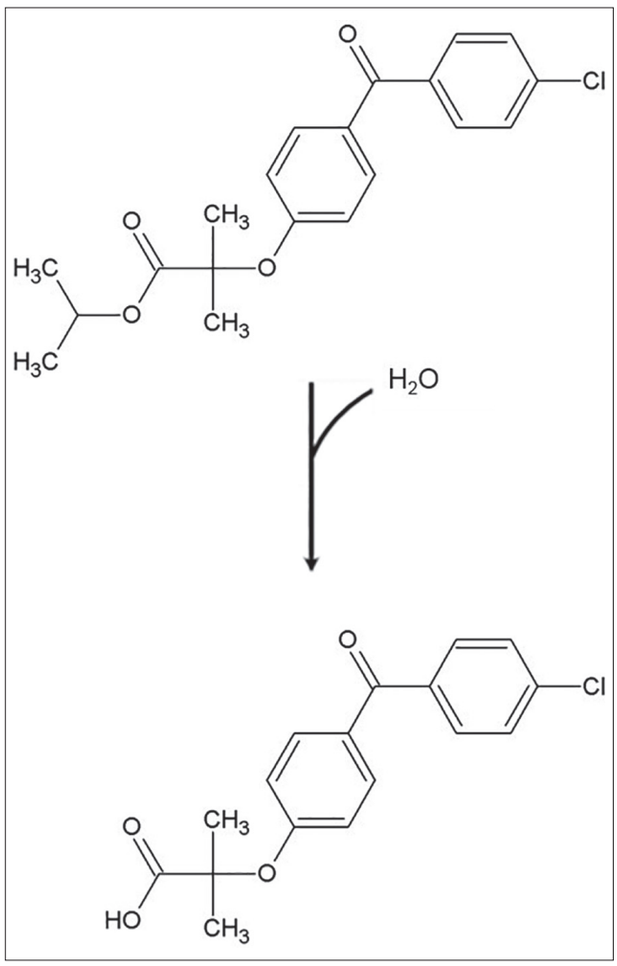

Fig. 6: Fenofibrate hydrolysis to fenofibric acid.

Different dissolution/permeation models have been introduced, but no standard experimental setup has been recognized (Kataoka et al. 2003; Motz et al. 2007; Ginski 1999; Zhou et al. 2014). The models differ in the dissolution vessel, the permeation chamber, and also in the applied permeation membrane. In order to define the key predictive parameters of dissolution/permeation models, this study compared three types of Caco-2 cell based models for their ability to distinguish between nanosized and micronized fenofibrate.

The first model was the IDAS1 chamber, which is designed according to the continuous dissolution/permeation model of Kataoka et al. (2003) and has successfully predicted differences between a variety of fenofibrate formulations previously (Buch et al. 2009). The model consists of a donor chamber of 8 mL and a receiver chamber of 5.5 mL, both stirred magnetically, and chambers separated by a Caco-2 cell monolayer. The experiments showed a higher concentration of permeated nanosized fenofibrate over the micronized drug, corresponding to the *in vivo* performance of both formulations in general (Maciejewski and Hillemann 2008). A comparison of the flux ratio of 2.6 in IDAS1 with the ratios of AUC (1.7) and C_{max} (4.4) calculated from literature data indicates that both systems found a better performance of the nanosized formulation over the micronized formulation, in clear accordance with the situation *in vivo* (Godfrey et al. 2011; Guivarch et al. 2004).

In order to evaluate the impact of the chamber proportions, the same experiment was performed in Ussing chambers, where the volume of both, donor and receiver chamber, was 5 mL. While Ussing chambers are generally stirred by gas lift, the donor chamber was additionally stirred magnetically. This model also depicted the superior performance of the nanosized fenofibrate formulation. The differences in volumes and stirring mechanism between Ussing and IDAS1 chambers affected the general outcome of the experiment only slightly. A higher flux of fenofibrate in the IDAS1 setup compared to the Ussing chambers could be due to more effective mixing of the receiver chamber in the IDAS1 setup. This may have removed the permeated fenofibric acid more effectively from the permeation membrane, generating an increased concentration gradient across the membrane, which ultimately increased the permeation.

In order to investigate the effect of membrane orientation in dissolution/permeation experiments, the outcome of the IDAS1 and Ussing chamber experiments, in which the Caco-2 cells are positioned vertically, was compared to the transwell setup that has a horizontal membrane orientation. The transwell model was not able to depict the better performance of the nanosized formulation,

the flux ratio was close to 1. Clearly, the *in vivo* situation was not predicted. In contrast to the vertical setups, which had stirring mechanisms inside each permeation chamber, the horizontal setup was stirred by gyrating the whole transwell plate. The hydrodynamics in the transwell chamber may have been insufficient for the dissolution of the formulations, which caused sedimentation of the drug particles directly upon the permeation membrane. In that case, the elimination of the dissolution process as the usual rate-limiting step for drug absorption, would explain the lack of differentiation between the formulations.

The Caco-2 cell based setups clearly showed the importance of membrane orientation and a much smaller impact of the chamber dimensions on the prediction of formulation effects by dissolution/permeation experiments.

While Caco-2 cells are the most common permeation membrane, excised rat intestinal sheets are an accepted additional model. Its application in dissolution/permeation experiments has been reported recently (Zhou et al. 2014). Unlike Caco-2 cells, rat intestine secrets mucus, which is a physiological barrier to drug particles. Its potential advantage in the predictivity of dissolution/permeation experiments for the evaluation of the effect of particle size reduction on drug absorption was therefore investigated.

The dissolution/permeation experiments with excised rat intestinal sheets did not discriminate between the formulations. Since the experiments were performed in the same Ussing chambers as the Caco-2 cell setup, hydrodynamics and other chamber specific issues can be ruled out as potential causes. It was striking that the permeation across excised rat intestinal sheets did not discriminate between the fenofibrate formulations, and was also much lower than across Caco-2 cells. This was unexpected due to several reasons. 1) The separate determination of dissolution and permeation behaviour stressed that the tested formulations primarily affected the dissolution process. Since the dissolution behaviour in the Ussing chambers sufficiently distinguished the formulations with mounted Caco-2 cells, the same setup with mounted excised rat intestine should be able to distinguish the formulation in a similar way. 2) The intestinal mucus layer was even expected to enhance the particle size distinction compared to Caco-2 cells. 3) The permeation across excised rat intestinal mucosa correlates well with human permeability data in general, and hence should have shown the superior behaviour of nanosized fenofibrate compared to the micronized formulation (Lennernäs 1998). 4) A comparison of both membranes usually shows higher permeation across excised rat intestinal mucosa (Artursson et al. 1993; Boisset et al. 2000; Menon and Barr 2003; Reis et al. 2013). However, the majority of these studies were performed with hydrophilic drugs, for which the permeation through the lipophilic membrane is rate limiting, whereas BCS class II drugs like fenofibrate permeate rapidly through the cell layer. As a hypothesis, the hydrophilic mucus layer may have functioned as an artificial additional barrier for the lipophilic drug. Even though the intestine physiologically secretes mucus, its preparation for the experiments stresses the tissue, which may increase the mucus secretion, resulting in an artificially thick mucus layer (Ungell 2002). Hence, the permeation through the enlarged mucus layer may have replaced the dissolution process as rate limiting step for fenofibrate absorption. Not only would such an effect explain the non-discrimination between the formulations in the dissolution/permeation experiments, it could also explain the much lower permeation of fenofibrate across excised intestinal sheets compared to Caco-2 cells. Another possible explanation is that the intestinal mucosa comprises underlying layers, which cannot be removed even with stripping, but have been shown to limit the permeation of hydrophobic compounds. Thus, for permeation across the intestinal mucosa *in vitro* a drug must cross these structures in addition to the epithelial layer, but *in vivo* it is essentially absorbed after crossing the epithelial layer. Permeation across Caco-2 monolayers mimics *in vivo* absorption more closely, because it represents only the epithelial layer, which is the rate-limiting barrier (Yamashita et al. 1997).

While not the object of the present study, the metabolism of fenofibrate was found different between Caco-2 cells and excised rat intestine. A reason for the different ratio of metabolized and unmetabolized fenofibrate in the setups may be the presence of different isozymes of intestinal carboxylesterases. The isozymes have different hydrolyzation capacity. Isozyme 2 of the human carboxylesterase (hCE2) has a higher capacity than hCE1 for compounds like fenofibrate, i.e. compounds with a large alcohol group and a small acyl group. Predominance of hCE2 in Caco-2 cells and of hCE1 in rat intestine could hence explain the lower fraction of fenofibric acid in rat intestine based setups.

In conclusion, the dissolution/permeation models IDAS1 and conventional Ussing chambers with mounted Caco-2 cells correctly predicted the differences in human exposure following oral administration of different sized fenofibrate formulations. The characteristics of the setups did not significantly affect the outcome, except when the setup utilized a horizontal membrane.

Dissolution/permeation experiments across excised rat intestinal sheets could not distinguish between the fenofibrate formulations. The identified key parameters in dissolution/permeation experiments were the type and orientation of the applied permeation membrane.

4. Experimental

4.1. Materials

Atenolol was obtained from Sigma-Aldrich (St. Louis. MO, USA). Antifoam B emulsion (AF), bovine serum albumin (BSA), fenofibrate, hydroxyethyl piperazinyl ethane sulfonic acid (HEPES), lecithin, sodium chloride (NaCl), and sodium taurocholate (NaTC) were purchased from Sigma-Aldrich (Steinheim, Germany). Hanks balanced salt solution (HBSS) was obtained from Life Technologies (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM), stable glutamine, penicillin/ streptomycin (PEST), nonessential amino acids (NEAA), and fetal bovine serum (FBS) were purchased from Biochrom (Berlin, Germany). FaSSIF powder was purchased from biorelevant.com (London, UK). Glucose was obtained from Fagron (Barsbüttel, Germany). Monosodium phosphate monohydrate was obtained from Merck (Darmstadt, Germany). Sodium hydroxide pellets were purchased from Riedel-de Häen (Seelze, Germany).

4.2. Fenofibrate formulations

For this study, tablets that contain fenofibrate in form of nanosized (Lipidil ONE) and micronized (Lipidil Ter, both Abbott, Hannover, Germany) particles were obtained from the university pharmacy in Mainz. Lipidil ONE tablets have a higher bioavailability than Lipidil TER tablets (Maciejewski and Hillemann 2008).

In order to avoid an impact of disintegration in the experiments, the tablets were mortared to obtain a powder. The respective powders are referred to as nanosized formulation and micronized formulation. Both formulations were applied at a concentration of 160 mg fenofibrate suspended in 250 mL of respective medium in all experiments.

4.3. Caco-2 cell experiments

Caco-2 cells were purchased from the European collection of cell cultures. Polycarbonate snapwells and transwells (1.12 cm^2 cell growth area) were purchased from Corning (Schiphol-Rijk, Netherlands).

Caco-2 cells were seeded on the 0.4 µm polycarbonate filters at a density of 100,000 cells/filter. Cells were grown in DMEM, which was supplemented with 1 % NEEA, 10 % FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. The medium was changed every other day. The cells were maintained at 37 °C, 5 % CO_2, and 90 % relative humidity. Passages 59-63 were used for the IDAS1 experiments, passages 44 - 46 for the Ussing chamber experiments (Klaus Mussler Scientific Instruments Aachen, Germany), and passage 62 for the transwell experiments. The experiments were performed 20 – 22 days after seeding.

4.4. Animal experiments

The study was carried out under the principles of animal care and approved by the animal ethical committee of Rhineland-Palatinate. Male Wistar Rats (11 weeks of age) were purchased at least one week prior to the experiments from Janvier Labs, France. The animals had free access to food (standard diet, Altromin) and tap water. The animals were anaesthetized with a mixture of ketamine and xylazine, the duodenum (1 - 10 cm distally from the pylorus) was removed, and the animals were decapitated immediately. The preparation of the excised intestinal segments was performed as previously described (Heinen et al. 2007) Briefly, the segments were rinsed twice with 10 mL ice-cold HBSS with 24.85 mM glucose, adjusted to pH 7.4 ($HBSSg_{7.4}$). Storage and preparation of the tissues was in ice-cold $HBSSg_{7.4}$ under continuous oxygenation with carbogen (95 % O_2, 5 % CO_2). The intestine was cut along the mesenteric border, segments containing Peyer's Patches were discarded, the serosa was stripped carefully and the segments were mounted in Ussing chambers. Intestinal segments with a transepithelial potential difference below 2 mV after the equilibration time were discarded.

4.5. Dissolution/permeation experiments

4.5.1 IDAS1 setup

The *in vitro* dissolution/absorption system 1 (IDAS1, Absorption Systems, Exton, Pennsylvania) is build according to the dissolution/permeation model developed by Kataoka et al. (2003). It consists of a donor chamber (8 mL), a receiver chamber (5.5 mL), and the connective Caco-2 cell monolayer (effective exposed area 1.77 cm^2). Integrated magnetic stirrers at 200 rpm stir both chambers. The donor medium consisted of $HBSSg_{6.5}$ with FaSSIF powder (biorelevant.com) and 100 µM atenolol, the receiver medium was $HBSSg_{7.4}$ with 4.5 % BSA. After equilibration of the membrane, the transfer of the drug formulations into the donor chamber and the simultaneous filling of donor and receiver chamber with the respective media started the experiment. Samples of 0.5 mL were taken from the receiver side after 15, 30, 60, 90, and 120 min, and replaced correspondingly.

4.5.2. Ussing chamber setup

The applied Ussing chambers consist of a donor and a receiver chamber (5 mL volume each), which are connected by a permeation membrane, continuously gas-stirred by carbogen, and in this study magnetic stirrers (Thermofisher Cimarec i Micro magnetic stirrers, Langenselbold, Germany) additionally stirred the donor chamber at 200 rpm. The permeation membrane consisted of either a monolayer of Caco-2 cells (effective exposed area 1.11 cm^2) or excised rat intestinal sheets (effective exposed area 0.67 cm^2) and was equilibrated before the start of the experiment.
The donor medium was $FaSSIF_{mod}$, its preparation was previously described (Khoshakhlagh et al. 2014). It consisted of HBSS with 24.85 mM glucose, 10 mM HEPES, 0.75 mM lecithin, 3 mM NaTC, and the pH was set to 6.5. $FaSSIF_{mod}$ was added to the formulations, vortexed for 5 s and immediately transferred to the donor chamber, which marked the start of the experiment. The receiver chamber was filled simultaneously, the receiver medium was $HBSSg_{7.4}$ with 4.5 % bovine serum albumin and 0.1 % Antifoam B emulsion. Samples of 0.5 mL were taken from the receiver side after 15, 30, 60, 90, and 120 min, and replaced correspondingly. Samples were stored at –80 °C or below until analysis.

4.5.3. Transwell setup

The setup and procedure of the dissolution/permeation experiments was the same as described for the Ussing chamber experiments but in transwells. The volume of the donor side was 0.5 mL and of the receiver side 1.5 mL, the effective exposed area was 1.12 cm^2. During the experiment, the transwells were kept in an incubator at 37 °C and 90 % relative humidity, on a rotating disk at 75 rpm. Samples of 0.5 mL were taken from the receiver side after 15, 30, 60, 90, and 120 min, and replaced correspondingly. Samples were stored at –80 °C or below until analysis.

4.6. Analysis

4.6.1. HPLC-MS

Drug and metabolite concentrations were determined using reverse-phase liquid chromatography (LC) with mass spectrometry (MS/MS) detection. Samples were prepared by diluting 50:50 with 100 % acetonitrile to precipitate the protein. The mixture was shaken for 5 min at 850 rpm, followed by centrifugation for 10 min at 3000 rpm. The supernatant was diluted 1:2 with water and injected into the HPLC system. The LC equipment was a Leap CTC HTS PAL autosampler (Leap Technologies, Carrboro, NC) and Agilent 1100 pumps (Agilent Technologies, Santa Clara, CA). Chromatographic separation was carried out at room temperature using a Thermo Hypersil BDS C18 30x2.1 mm i.d., 3 mm, column with a guard column (Thermo Fisher Scientific, Waltham, MA). The mobile phase buffer was 25 mM ammonium formate buffer, pH 3.5 (buffer). Mobile phase A contained 90% water and 10% buffer and mobile phase B contained 90% acetonitrile and 10% buffer. Separation conditions were: 100% A at time 0, to 0% at 1.5 min, and 100% A at 2.1 min (total run time was 3.5 min) a flow rate of 250 mL/min. The injection volume was 10 mL. MS/MS was performed on a PE Sciex API4000 triple quadrupole mass spectrometer in the MRM mode, using a turbo ionspray interface (AB Sciex, Framingham, MA). Briefly, the transitions for fenofibrate and its internal standard (fenofibrate-D6) were 361.00/233.00 and 367.20/234.10, respectively, and for fenofibric acid and its internal standard (fenofibric acid-D6) were -317.00/-231.00 and -323.00/-231.20, respectively. The lower limit of quantitation was 15 nM for both compounds. The standard curves for both compounds had good linearity between 15 and 5000 nM, as indicated by correlation coefficients (R2) values of 0.9997 and 0.9999 for fenofibrate and fenofibric acid, respectively. The accuracy for all standards and 3 levels of quality control samples were in the 85-115% range.

4.6.2. Data analysis

The low dose dissolved during the dissolution experiments excluded analysis of the profiles by the similarity factor f2. For statistical evaluation of the dissolution chambers, the dissolved amount was compared by student's t-test at each time point. The difference was considered significant when $P < 0.05$.
The permeated amount of drug in the dissolution/permeation experiments was calculated as the sum of detected fenofibrate and fenofibric acid. The formulations were compared by Student's t-test of the dissolved or permeated amount. The difference was considered significant when $P < 0.05$. The fluxes of the formulations were calculated according to Eq. (1)

$$Flux = \frac{dQ}{dt} \times \frac{1}{A} \quad (1)$$

where dQ/dt is the rate of transport across the membrane and A is the exposed area of the mounted tissue.
The apparent permeability P_{app} of atenolol served as control of integrity. Caco-2 monolayers were considered intact with permeability values of less than 0.5×10^{-6} cm/s.[9] P_{app} was calculated according to Eq. (2),

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A \times c_0} \quad (2)$$

where c_0 is the donor concentration of atenolol.
The variability is expressed as standard error of the mean (SEM) throughout the paper unless otherwise stated.
All figures were made using Origin 7.5 (Northampton, Massachusetts).

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Conflicts of interest: None declared.

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