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Influence of lipid lowering fibrates on P-glycoprotein activity *in vitro*

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Abstract

Statin/fibrate combinations are frequently used to treat mixed dyslipidemia. However, these combinations may cause life-threatening drug interactions (e.g. rhabdomyolysis) possibly induced by modifications of cytochrome P450 isozyme activities. Some statins are also transported by P-glycoprotein (Pgp) and may act as inhibitors of this drug efflux pump. So far, nothing is known about possible Pgp modulating effects of fibrates. We tested whether gemfibrozil, fenofibrate, fenofibric acid, and bezafibrate inhibit Pgp *in vitro* using a calcein acetoxymethylester (calcein-AM) uptake assay and confocal laser scanning microscopy with bodipy-verapamil as substrate in L-MDR1 cells, which overexpress human Pgp. In uptake assays in cells with (L-MDR1) and without (LLC-PK1) human Pgp we also investigated whether these compounds are transported by Pgp. Intracellular concentrations were measured by liquid chromatography tandem mass spectrometry. Of the tested fibrates, only fenofibrate increased calcein-AM uptake into cells indicating an inhibition of Pgp mediated transport by this compound. The potency of fenofibrate (mean ± SD: 7.1 ± 3.2 μM), evaluated by calculating the concentration needed to double baseline fluorescence (f2), was similar to that of simvastatin (5.8 ± 1.5 μM), lovastatin (10.1 ± 1.0), and verapamil (4.7 ± 0.8 μM). For simvastatin and fenofibrate Pgp inhibition was confirmed with confocal laser scanning microscopy. Fenofibrate, fenofibric acid, gemfibrozil, and bezafibrate showed no difference in the cellular uptake between LLC-PK1 and L-MDR1, indicating that the tested fibrates are not Pgp substrates. In conclusion, this study demonstrates that fenofibrate inhibits Pgp *in vitro* with a potency similar to simvastatin.

Keywords: Fibrates; P-glycoprotein; *In vitro*; Drug interactions; Calcein-acetoxymethylester; Inhibition

1. Introduction

Lipid lowering fibrates are an established class of drugs for the treatment of hypertriglyceridemia. Combined with 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), which are used primarily to treat hypercholesterolemia, they represent an attractive approach for the treatment of mixed dyslipidemia because of their different mechanisms of action. While statins reduce cholesterol synthesis fibrates increase catabolism of triglyceride rich lipoproteins and increase HDL-cholesterol. For both, statins and fibrates, drug-induced myopathy is a rare but well documented adverse reaction [1] and the risk for progression to life-threatening rhabdomyolysis is increased when statins and fibrates are used in combination [1–3]. The incidence of these interactions depends on the individual drug and the drug combination used as shown in an analysis of all fatal cases of rhabdomyolysis reported to the American Food and Drug Administration (FDA) until June 2001. In this series no case of fatal rhabdomyolysis for fluvastatin was reported whereas this rate was 3.16 per million cerivastatin prescriptions [3]. Twelve of the 31 cases reported to the FDA occurred during combined use of cerivastatin and gemfibrozil. The most common source for such drug–drug interactions is thought to be the cytochrome P450 (CYP) system. Whereas pravastatin is not metabolized by the CYP system simvastatin, lovastatin, and atorvastatin are mainly metabolized by the CYP3A4

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Abbreviations: APCI, atmospheric pressure chemical ionization; calcein-AM, calcein-acetoxymethylester; CYP, cytochrome P450; DMSO, dimethyl sulfoxide; ESI, electrospray ionization; f2, concentration needed to double baseline fluorescence; HBSS, Hanks' balanced salt solution; HHBSS, HBSS supplemented with 10% HEPES; IC_{50}, concentration inducing half maximal effect; LC/MS/MS, liquid chromatography tandem mass spectrometry; LDH, lactate dehydrogenase; MRP, multidrug resistance-associated protein; Pgp, P-glycoprotein.

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isoform, fluvastatin by CYP2C9, and cerivastatin by CYP3A4 and CYP2C8 [4,5]. Also gemfibrozil, fenofibrate, and bezafibrate are metabolizes via CYP3A4 [6]. Even though gemfibrozil elevates the risk to develop statin-induced muscle disorders it does not inhibit CYP3A4 in human liver microsomes suggesting a different mechanism of drug interaction [7]. Another possible interaction site is Pgp, a member of the superfamily of ATP binding cassette (ABC) transporters. This efflux transporter is mainly expressed at the blood–brain barrier [8] and the intestinal barrier [9] and participates in the renal and biliary elimination of drugs [10,11]. It prevents intracellular drug accumulation by actively removing compounds from the cell. Therefore, Pgp plays a role in absorption, distribution, and excretion of certain drugs and may be an important site of drug–drug interactions. Indeed, *in vitro* several statins are inhibitors of Pgp [12–15], and many of them are also substrates of the efflux pump [13,16] suggesting that drug interactions might also occur at the level of Pgp. Thus far, the interaction of fibrates with Pgp has not been evaluated.

The aim of the present study was to assess the Pgp modulating effect of fibrates and to compare it with the known Pgp inhibitor effects of simvastatin and lovastatin. Therefore, we investigated the Pgp inhibitory potency of lipid lowering fibrate derivatives *in vitro* in a porcine kidney epithelial cell line overexpressing human Pgp (L-MDR1) [17] and the corresponding parental cell line (LLC-PK1) as a negative control without the human ABC transporter. In particular we studied the lipid lowering active parent compounds gemfibrozil and bezafibrate, and the inactive prodrug fenofibrate with its active metabolite fenofibric acid. For this assay we used the fluorogenic calcein-AM as Pgp substrate and confirmed the results using a different technique (confocal laser scanning microscopy) with an independent Pgp substrate (bodipy-verapamil). Furthermore, intracellular drug accumulation of each fibrate in L-MDR1 and LLC-PK1 cells was measured directly by LC/MS/MS to assess whether test compounds are Pgp substrates.

2. Material and methods

2.1. Drugs

Gemfibrozil, fenofibrate, bezafibrate, lovastatin, loperamide, and verapamil hydrochloride were obtained from Sigma-Aldrich. Vincristine sulfate was purchased from Calbiochem and LY335979 was a generous gift from Eli Lilly Co.

2.2. Solvents, chemicals, and cell culture accessory

Methanol Uvasol, hexane Uvasol, methylene chloride Uvasol, acetonitrile LiChrosolv, water LiChrosolv, formic acid for analysis, hydrochloric acid, and sodium hydroxide were from Merck. Cell culture medium M199, fetal calf serum (FCS), glutamine, 100 U/mL penicillin with 100 μg/mL streptomycin sulfate, HBSS, HEPES, and versene were from Invitrogen. Collagen-R was obtained from Serva. DMSO and Triton X-100 were purchased from AppliChem, calcein-AM from MoBiTec, 48-well plates from Falcon, and 96-well plates and 80 cm^2 culturing bottles from Nunc.

2.3. Isolation procedures

2.3.1. Simvastatin

According to Bogman *et al.* [14] 50 tablets Zocor® forte containing 40 mg simvastatin from Merck Sharpe & Dohme Limited were mortared and stirred 2 hr with 100 mL of an extraction solution containing methanol, hexane, and methylene chloride in equal volumetric parts. The extraction solution was filtered and evaporated to dryness under a stream of nitrogen at 50°. The remaining residue was weighed and redissolved in acetonitrile/water and deuterated DMSO (d_3-DMSO). Subsequently, the solutions were analyzed to determine purity and identity by HPLC and spectroscopic measures.

2.3.2. Fenofibric acid

An amount of 1.06 g of fenofibrate was dissolved in a mixture of 8 mL acetonitrile and 4 mL water. After addition of 40 pellets sodium hydroxide the mixture was heated to 90° in a water bath for 90 min. After 1 hr, 2 mL water and 40 mL 1 M hydrochloric acid were added. For separating the two phases the mixture was allowed to cool down and further 20 mL hydrochloric acid were added. Fenofibric acid was precipitated from the aqueous phase by acidification with 1 M hydrochloric acid (200 mL). The precipitate was centrifuged for 10 min at 3000 *g* and was dried at 90°. The remaining residue was also weighed and redissolved in acetonitrile/water and d_3-DMSO to determine purity and identity.

2.4. Purity and identity

Purity of isolated simvastatin and fenofibric acid was determined by HPLC/UV (215 and 254 nm) using a pump gradient program (100% aqueous eluent to 100% organic eluent). The purity was >99% for either compound. Identity of both isolated compounds was confirmed by ESI LC/MS/MS in the daughter ion scanning mode and nuclear magnetic resonance (NMR) spectroscopy (one-dimensional ^1H and ^{13}C, as well as two-dimensional HH-Cosy and CH-Cosy).

2.5. Stock solutions

Stock solutions of test compounds were prepared following the manufacturers' instructions. All compounds

were dissolved in DMSO. The DMSO concentration in the assays never exceeded 1% (v/v), a concentration that was found not to influence the results of the assay in pilot experiments.

2.6. Calcein uptake assay

The assay was used to determine the Pgp inhibitor potency of the test compounds and was performed as previously described [18] with calcein-AM in a final concentration of 0.5 μM.

Quenching test and cytotoxicity assay were performed for each test compound as previously described [18,19].

2.7. Confocal laser scanning microscopy

Intracellular accumulation of the dye bodipy-verapamil in the presence of the test compounds was analyzed with a DM IRE 2 TCS SP II confocal laser scanning microscope from Leica as described earlier [19]. Bodipy-verapamil instead of calcein-AM was chosen as a substrate to confirm the interaction with calcein with an independent Pgp substrate. In addition, bodipy-verapamil provides the advantage to be more resistant to photobleaching effects caused by the laser.

2.8. Uptake assay

To evaluate the substrate characteristics L-MDR1 and LLC-PK1 cells were incubated with the test substance (100 μM for gemfibrozil, fenofibric acid, and bezafibrate and 25 μM for fenofibrate) with and without Pgp inhibitor (LY335979). To demonstrate the suitability of the assay, loperamide (1 μM) was used as a Pgp substrate in a separate experiment in both cell lines. One day before performing the assay culture medium was replaced by vincristine-free medium. The assay was conducted in collagen-coated 48-well microtiter plates (150,000 cells/cm^2). All incubation steps and cell lysis were performed at room temperature on a rotary shaker at 450 rpm. The cells were washed with prewarmed HHBSS and preincubated for 1 hr with HHBSS. Subsequently, HHBSS as a control or LY335979 (final concentration 5 μM in uptake experiments with fibrates and 1 μM for uptake experiments with loperamide) as a Pgp inhibitor was added for 15 min. The test compound was applied and the supernatant was detached after 2, 10, 30, or 60 min (sextuplet determination). The cells were washed twice with precooled (4°) HHBSS and lyzed with 1% Triton X-100 within 30 min. After adding internal standard the cell lysates were directly analyzed by LC/MS/MS.

2.9. Analytical methods

For the quantification of intracellular drug concentrations in the uptake assays methods depending on LC/MS/MS were developed. The mass spectrometric analyses were performed on a triple stage quadrupole instrument (TSQ 7000, Thermo Finnigan). Sample admission was done by an autosampler Type 232XL from Gilson Abimed and an LC gradient pump (P4000 Thermo Finnigan) was used with a degasser. Chromatography was performed on a Kromasil RP18 CC 125/3; 100-3.5 analytical column (Macherey und Nagel) using an isocratic eluent (flow 0.35 mL/min) consisting of water with 1% formic acid (eluent A) and acetonitrile (eluent B) in different volumetric parts depending on the drug (Table 1). For detection of fenofibrate, fenofibric acid, bezafibrate, and loperamide [20] single reaction monitoring (MS/MS) mode with electrospray ionization (ESI, 4.5 kV, Sheath/Aux gas N_2) and argon as collision gas (3 mbar) was used. Gemfibrozil was detected in single ion monitoring mode with atmospheric pressure chemical ionization (APCI, 5.0 μA, 500°, Sheath/Aux gas N_2). Details of the LC and mass spectrometric parameters are shown in Table 1.

The calibration range for gemfibrozil, fenofibric acid, and bezafibrate was 0.1–200 μM, for fenofibrate 0.025–50 μM and 0.0025–0.5 μM for loperamide. The lower limit of quantification was 0.1 μM for gemfibrozil, fenofibric acid, and bezafibrate, 0.025 for fenofibrate, and 0.0025 μM for loperamide.

2.10. Statistical analysis

Due to cytotoxic influences, plateau effects and thus IC_{50} values (the concentration leading to half-maximal inhibition of the calcein-AM transport) could not be determined for all test compounds. Based on the concentration–response curve we therefore also calculated the concentration needed to double baseline fluorescence (f2) according to Weiss *et al.* [18]. Unless indicated otherwise data are expressed as mean ± SD. For the f2 values *P*-values were

Table 1
LC/MS/MS parameters for quantification of the intracellular drug uptake

Analyte	Eluent, %A/%B	Rt (min)	Parent mass (*m/z*)	Center mass (*m/z*)	Ionization	Collision energy (V)
Gemfibrozil	30/70	2.01	249.2	–	APCI negative	–
Fenofibrate	30/70	3.69	316.2	233.1	ESI positive	22
Fenofibric acid	50/50	3.45	316.9	230.8	ESI positive	22
Bezafibrate	60/40	3.31	362.2	316.1	ESI positive	28
Loperamide	55/45	2.55	477.2	266.3	ESI positive	28

A = 1% formic acid; B = acetonitrile; Rt = retention time.

determined by ANOVA with Dunnett's multiple comparison test for *post hoc* pairwise comparison with the control results obtained with verapamil. For the uptake assay, *P*-values were determined by ANOVA with Bonferroni's multiple comparison test for *post hoc* pairwise comparison. *P*-values for the experiments with the confocal laser scanning microscope were calculated by one-tailed *t*-test. All statistical analysis was performed with GraphPad InStat, version 3.05, GraphPad Software. A *P*-value of ≤ 0.05 was considered significant.

3. Results

3.1. Pgp inhibitor potency

None of the test substances showed any quenching effect in the concentration range tested on the fluorescence of calcein and no autofluorescence at the excitation wavelength used to measure calcein fluorescence (485 nm) was observed. Moreover, no self-quenching effects of calcein were observed in the concentration range applied.

The two paradigm Pgp inhibitors verapamil and LY335979 and the fibrates were not cytotoxic in the concentration range used in the assays whereas the statins were cytotoxic in concentrations higher than 100 μM (lovastatin) and 35 μM (simvastatin) in the LDH detection assay and simvastatin as well as in confocal laser scanning microscopy (manifesing in deformations of the cells).

Of all tested compounds simvastatin, lovastatin, and fenofibrate showed an inhibitory effect on Pgp in the calcein assay. In contrast to fenofibrate, where a plateau in the concentration–response curve was reached (Fig. 1), for simvastatin and lovastatin (Fig. 2) this was impossible (due to cytotoxic effects), and thus no IC_{50} could be calculated. On the basis of f2-values the potency of fenofibrate was similar to the potency of the statins and verapamil, a well-known Pgp inhibitor (Table 2). Comparing the IC_{50} values, fenofibrate was even 1.5 times more potent than verapamil ($P < 0.01$). Except for simvastatin, which showed a minor effect also in LLC-PK1 cells, all other compound tested had no effect in the control cell line.

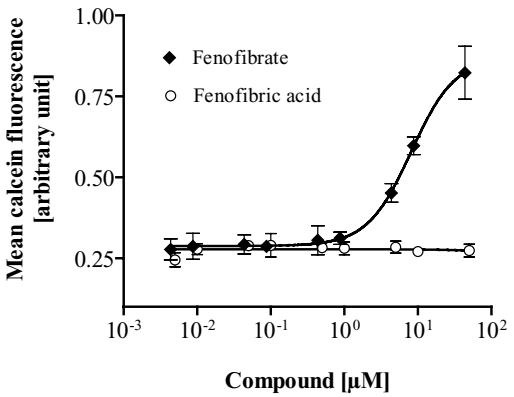

Fig. 1. Concentration response curve of fenofibrate and fenofibric acid obtained with calcein assay in L-MDR1 cells. Data are expressed as mean ± SD for N = 8 wells.

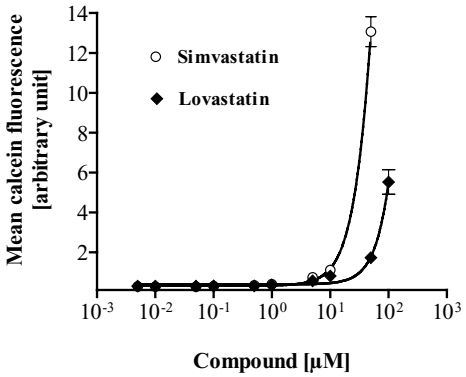

Fig. 2. Concentration response curve of simvastatin and lovastatin obtained with calcein assay in L-MDR1 cells. Data are expressed as mean ± SD for N = 8 wells.

For simvastatin and fenofibrate the results obtained in the calcein assay were further evaluated with an independent assay based on confocal laser scanning microscopy with bodipy-verapamil as a substrate confirming the Pgp inhibitor activity of both compounds (Fig. 3). In this assay fenofibrate significantly increased basal fluorescence by 1.46 ± 0.12 (25 μM) and 1.69 ± 0.19 (50 μM) in L-MDR1 cells ($P < 0.05$) whereas in the control cell line LLC-PK1 no effect was observed (Fig. 3). Simvastatin (25 μM) significantly increased basal fluorescence in L-MDR1 cells by 1.79 ± 0.34 ($P < 0.05$) (Fig. 4), but also in LLC-PK1 cells by 1.19 ± 0.06 ($P < 0.05$). The effect seen in LLC-PK1 cells was clearly smaller than in the overexpressing cell line ($P < 0.05$).

3.2. Pgp substrate characteristics

To investigate whether the fibrates are transported by Pgp an uptake assay in LLC-PK1 and L-MDR1 cells was performed. First, the suitability of the uptake assay for

Table 2
Inhibition of Pgp by lipid lowering fibrates and typical Pgp inhibitors in L-MDR1 cells

Test compound	N	f2 (μM)	IC_{50} (μM)
Gemfibrozil	4	n.d.	n.d.
Fenofibrate	4	7.1 ± 3.2	5.83 ± 2.3*
Fenofibric acid	3	n.d.	n.d.
Bezafibrate	4	n.d.	n.d.
Simvastatin	3	5.8 ± 1.5	n.d.
Lovastatin	3	10.1 ± 1.0**	n.d.
Verapamil	3	4.7 ± 0.8	20.0 ± 3.1
LY335979	4	0.02 ± 0.01**	0.14 ± 0.06*

N = number of experiments, each performed in octuplet. Values are given as mean ± SD. *P*-values are determined by ANOVA with Dunnett's multiple comparison test for *post hoc* pairwise comparison of the results with the verapamil control. f2 = concentration needed to double baseline fluorescence calculated according to [21]; n.d.: not definable.

*$P < 0.01$; **$P < 0.05$.

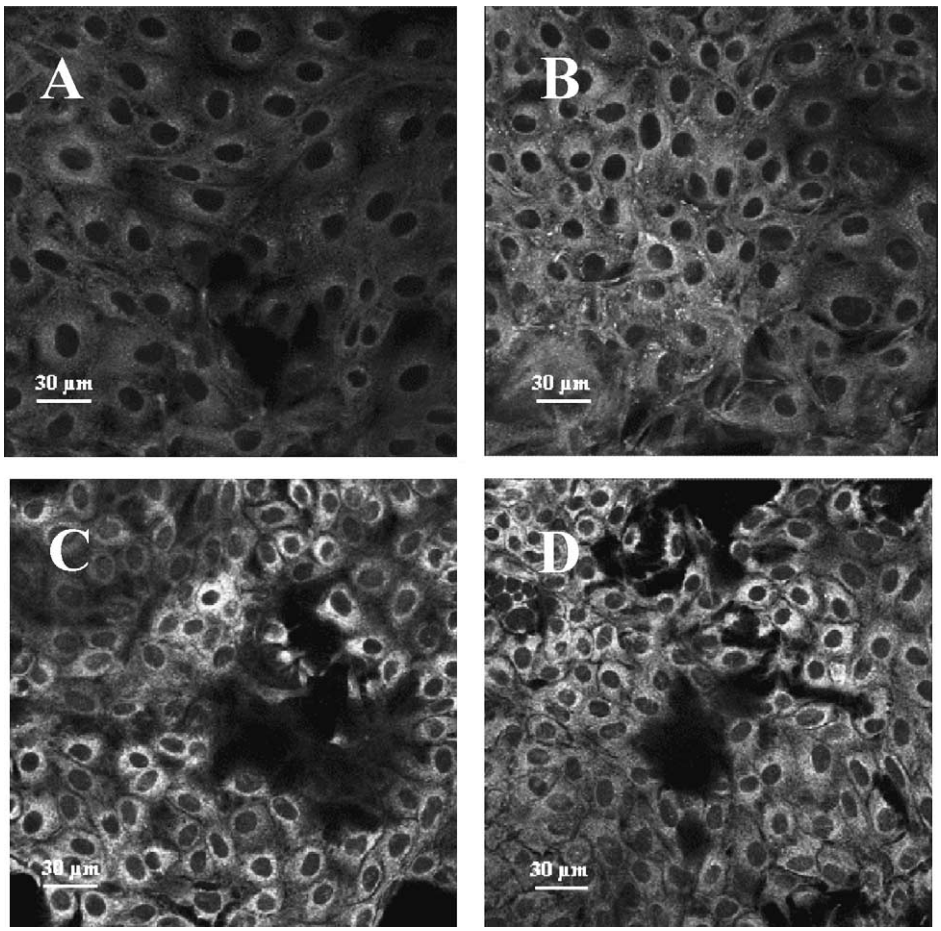

Fig. 3. Confocal laser scanning microscopy: bodipy-verapamil uptake into L-MDR1 (A and B) and LLC-PK1 (C and D) cells. On the left: without fenofibrate. On the right: after exposure for 20 min with fenofibrate (50 µM) as Pgp inhibitor.

evaluating Pgp substrate characteristics was tested using the known Pgp substrate loperamide. The loperamide uptake was significantly higher in the parental cell line compared to L-MDR1 cells ($P < 0.01$) (Fig. 5A). Pgp inhibition with LY335979 completely abolished drug resistance of L-MDR1 cells leading to similar concentration–time profiles as in the parental cell line ($P < 0.001$, compared to L-MDR1 cells without LY335979). In LLC-PK1 cells LY335979 had no statistically significant effect on the loperamide uptake (Fig. 5A).

In this assay there was no difference between the two cell lines in the intracellular uptake of fenofibrate (Fig. 5B),

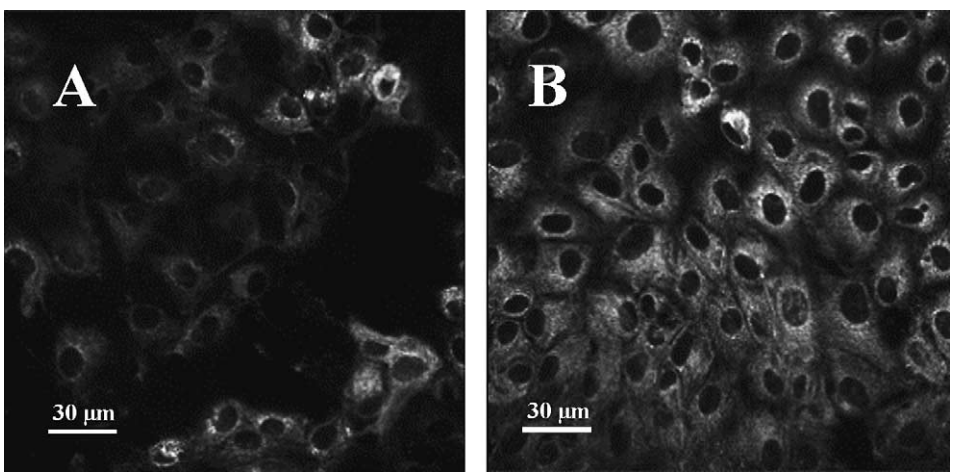

Fig. 4. Confocal laser scanning microscopy: bodipy-verapamil uptake into L-MDR1 (A and B) cells. On the left: without simvastatin. On the right: after exposure for 20 min with simvastatin (25 µM) as Pgp inhibitor.

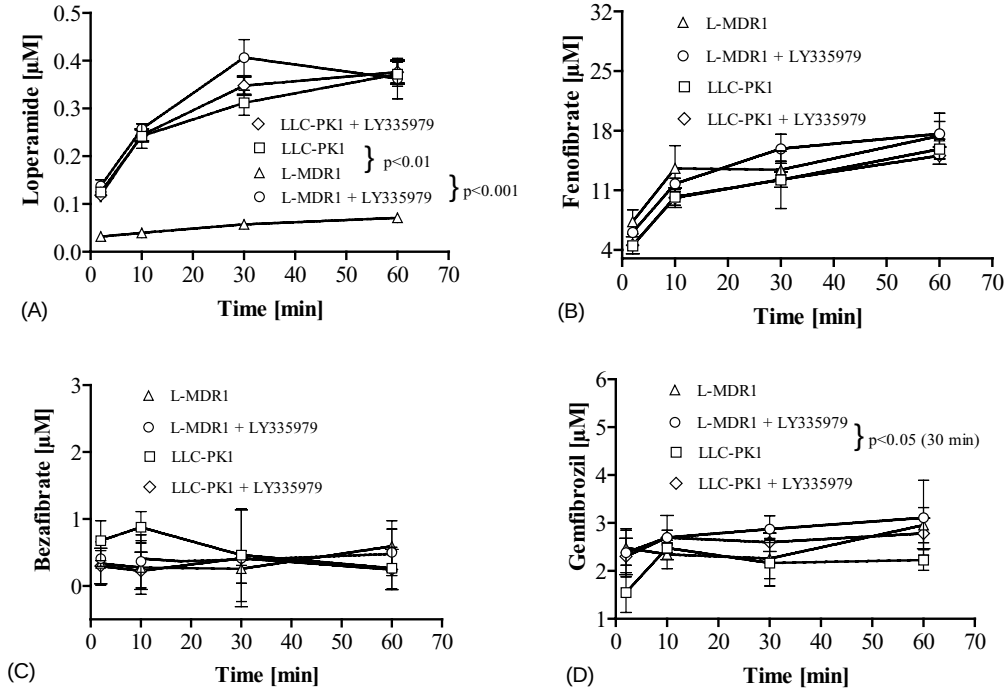

Fig. 5. Uptake assay of loperamide (1 µM) (A), fenofibrate (25 µM) (B), bezafibrate (100 µM) (C), and gemfibrozil (100 µM) (D) in LLC-PK1 cells and in L-MDR1 cells with and without Pgp inhibition by LY335975 (1 µM for the uptake with loperamide; 5 µM for the uptake with the fibrates). Data are shown as mean ± SD for N = 6 wells. *P*-values were determined by ANOVA with Bonferroni's multiple comparison test for *post hoc* pairwise comparison.

bezafibrate (Fig. 5C), gemfibrozil (Fig. 5D) or fenofibric acid (data not shown).

4. Discussion

The activity of the efflux transporter Pgp affects the pharmacokinetics of many drugs and contributes to numerous pharmacokinetic drug–drug interactions [21]. Hitherto, the role of Pgp for the bioavailability, distribution, and excretion of lipid lowering fibrates has not been investigated. Especially with statins severe interactions have occurred. A well known example is the increased risk for rhabdomyolysis emerging from the combination of cerivastatin with gemfibrozil, which recently lead to the withdrawal of cerivastatin from the European and US market. Interestingly the incidence of these adverse drug reactions depends on the individual statin and fibrate and the particular combination chosen. For instance in the published cases of rhabdomyolysis after a fibrate/statin combination between 1989 and July 2000 gemfibrozil was incriminated in each case [22]. Furthermore, about 25% of the rhabdomyolysis related mortality with cerivastatin was associated with cerivastatin–gemfibrozil combination therapy [23]. Whereas part of this interaction is likely caused by inhibition of CYP2C8 [7,24] cerivastatin and many other statins also interact with Pgp [13–16,25]. A further explanation for the accumulation of statin metabolites during co-administration of fibrates could therefore be an interaction at the level of Pgp by which statins are actively transported [13,16].

The aim of this *in vitro* study was to elucidate whether fibrates are transported by Pgp and whether they inhibit this drug transporter. An evaluation of both properties was necessary because some Pgp substrates do not act as Pgp inhibitors (e.g. digoxin) [18,26,27] and some inhibitors are not transported [27]. All assays were performed with the cell line L-MDR1 overexpressing human Pgp and the corresponding native cell line LLC-PK1 without human Pgp as a control. Thus, effects only seen in L-MDR1 cells can be attributed to functional human Pgp. Except simvastatin all compounds tested had no effect in LLC-PK1 cells. The fact, that simvastatin not only increases the uptake of bodipy-verapamil and of calcein-AM in L-MDR1 cells, but also revealed a minor effect in LLC-PK1 cells and of calcein-AM in LLC-PK1 (data not shown) indicates that simvastatin not only inhibits Pgp, but possibly also other transporters like multidrug resistance-associated proteins (MRPs).

In agreement with earlier investigations simvastatin was more potent an inhibitor of Pgp than lovastatin [14,15]. Moreover, our study demonstrates that gemfibrozil and bezafibrate do not interact with Pgp, whereas fenofibrate acts as a Pgp inhibitor. In line with the model established by Seelig [28], all fibrates contain electron donor patterns, which might interact with Pgp. However, whereas the other fibrates are anionic and hydrophilic compounds which are normally weak Pgp substrates if at all [10] only fenofibrate is lipophilic and neutral which predicts high affinity to Pgp. Therefore, our finding that fenofibrate inhibits Pgp could be expected. It is, however, surprising that fenofi-

brate appeared not to be transported, because no difference between the uptake into the parental cell line (LLC-PK1) and the overexpressing cell line (L-MDR1) was found (Fig. 5B). Therefore, similar to verapamil, fenofibrate might exhibit a high intrinsic permeability and pass through the cell membrane faster than it is removed by Pgp [27]. In this case, the transport will not be detectable in an uptake assay.

In agreement with the lack of Pgp inhibition there was no difference in the uptake of gemfibrozil and bezafibrate between the two cell lines (Fig. 5C and D), indicating that these compounds are not transported by Pgp.

The present results implicate that the interactions occurring between drugs and the fibrates gemfibrozil and bezafibrate, as well as the known differences in their interaction pattern [29] are not due to interactions with Pgp. We have not studied all known fibrates and not all of their metabolites and hence cannot rule out that some of them might interact with human Pgp. However, a major active metabolite (fenofibric acid) and the lipid lowering parent compounds (gemfibrozil, bezafibrate) clearly did not interact with Pgp.

Based on our results only for fenofibrate an *in vivo* interaction on the level of Pgp is conceivable. Because fenofibrate proved to be an inhibitor of Pgp it appears possible that the bioavailability of statins and other Pgp substrates will increase during co-administration of fenofibrate. However, if occurring at all these interactions will be limited to gastrointestinal Pgp because fenofibrate is undergoing a nearly complete presystemic metabolism [6,30]. Whether this interaction occurs and whether it is clinically relevant has to be elucidated *in vivo*. However, one of the very few interaction studies performed with fenofibrate suggests that it does not affect the pharmacokinetics of oral cyclosporine A [31] whose absorption is modulated by intestinal Pgp [32].

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