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HMG-CoA reductase inhibitors and P-glycoprotein modulation

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1 Five 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), (e.g. atorvastatin, fluvastatin, lovastatin, pravastatin and simvastatin), were investigated for their ability to reverse P-glycoprotein (P-gp) mediated rhodamine 123 (R123) transport in a murine monocytic leukaemia cell line that over-expresses the multi-drug resistance protein 1a/b (mdr1a/1b).

2 P-gp modulation was studied by a fluorimetric assay and confocal microscopy by means of R123 efflux and uptake experiments, respectively.

3 Atorvastatin acid, methyl ester and lactone, lovastatin lactone and simvastatin lactone inhibited R123 transport in a concentration-dependent manner. Lovastatin acid, simvastatin acid, fluvastatin and pravastatin did not show a significant inhibition of the R123 transport in our cell system. Atorvastatin methyl ester and lactone showed the highest affinities for P-gp and results were comparable for both methods.

4 In conclusion, monitoring of R123 transport in living cells by confocal microscopy in addition to fluorimetric assay is a sensitive tool to study P-gp affinity in drug screening that is especially useful for early phases of drug development.

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Keywords: Statins; P-glycoprotein; rhodamine; *in vitro*; confocal microscopy; drug interactions; HMG-CoA reductase inhibitors

Abbreviations: ATO, atorvastatin; cMOAT, canalicular multispecific organic anion transporter; CYP450 3A4, cytochrome P450 3A4; DMSO, dimethyl sulfoxide; FLU, fluvastatin; HBSS, Hank's balanced salt solution; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; IP, inhibitory potency; LOV, lovastatin; Log P, log of octanol/water partition coefficient; MDR, multi-drug resistance; OATP, organic anion transporter; P-gp, P-glycoprotein; PI, propidium iodide; PRA, pravastatin; R123, rhodamine 123; SIM, simvastatin; t_{50}, time needed to reach the half-maximal effect

Introduction

P-glycoprotein (P-gp, mdr1-gene product), a member of the superfamily of ATP-binding cassette (ABC) transporters, has been intensively examined because of its multi-drug resistance (MDR) phenotype in oncology. This multi-drug transporter prevents the intracellular accumulation of anti-cancer drugs by actively removing them from the cell membrane before they reach their intracellular targets.

In the body, P-gp is widely expressed on the luminal surface of capillary endothelial cells in brain and testis, adrenal cortex, brush border membrane of the proximal renal tubule epithelium, canalicular membranes of hepatocytes, mucosa of the small and large intestine, pancreatic ductules, endometrium of gravid uterus and placenta (Ambudkar *et al*., 1999). Strong morphological and genetic evidence exist demonstrating a role of P-gp in absorption, distribution and excretion of certain hydrophobic, amphiphatic drugs and xenobiotics in mice and probably in humans (Schinkel, 1997). This may also lead to pharmacokinetic drug–drug interactions in human as described by different authors (Boyd *et al*., 2000; Hunter & Hirst, 1997; Yu, 1999). Thus two drugs that are transported by P-gp could compete for the transporter and as a result increase oral absorption, decrease hepatic/renal excretion and/or alter distribution into tissues where there is expression of this protein.

It is of interest to note that the cytochrome P450 system, especially the isoform P450 3A, has an overlapping substrate specificity to that of P-gp (Kim *et al*., 1999; Wacher *et al*., 1995).

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) represent an established class of drugs for the treatment of hypercholesterolaemia, with potentially fatal adverse events (such as rhabdomyolysis). The lipophilic drugs lovastatin, simvastatin, atorvastatin and fluvastatin are metabolized *via* the cytochrome P450 system in the liver and the gut, making them subject to potential interactions with concomitantly administered drugs that are competing for metabolism *via* this system (Christians *et al*., 1998). Pravastatin is water-soluble and does not undergo metabolism *via* CYP450 to any significant extent. Both lovastatin and simvastatin are inactive lactone pro-drugs and are converted by hydrolytic enzymes in plasma and liver to the active acid form (Tang & Kalow, 1995) whereas atorvastatin, fluvastatin and pravastatin are administrated in their active acid form (Transon *et al*., 1996). All statins included in this study, except pravastatin, show a high hepatic extraction ratio. Furthermore it is known that fluvastatin (Lennernäs & Fager, 1997; Lindahl *et al*., 1996),

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lovastatin (Halpin *et al*., 1993) and simvastatin metabolites (Cheng *et al*., 1994) are excreted into bile, and that pravastatin has an important glomerular filtration and tubular secretion.

Membrane transport of these compounds into different tissues can occur by passive and/or carrier-mediated processes. Implication of the latter processes for liver selectivity or elimination has been described or hypothesized earlier for some statins (Boyd *et al*., 2000; Christians *et al*., 1998; Hsiang *et al*., 1999; Lindahl *et al*., 1996; Tamai *et al*., 1997; Wu *et al*., 2000; Yamazaki *et al*., 1997).

Numerous factors contributing to the risk for adverse drug interactions with statins have been reported recently (Bottorff, 1999; Christians *et al*., 1998; Corsini *et al*., 1999), and should be considered when patients are receiving additional drugs. Knowledge about the differences in the adverse drug interaction profile of statins is an important determinant of safety in long-term therapy of hypercholesterolaemia.

In vitro screening methods for detection of P-gp affinity are numerous and include primary cell culture systems (Drewe *et al*., 1999), fresh tissue (Fricker *et al*., 1999), over-expressing cell lines (Pourtier-Manzanedo *et al*., 1992), membrane vesicles (Shapiro *et al*., 1997), purified P-gp on h.p.l.c. columns (Zhang *et al*., 2000).

In the present study, we developed an *in vitro* fluorimetric assay and a confocal microscopy method to study the ability of five statins and their derivates for modulation of transport of a fluorescent P-gp substrate rhodamine 123 (R 123). As cell system, a murine mdr1a/1b over-expressing monocytic leukaemia cell line and its parental analogue (Boesch *et al*., 1991) was used.

Methods

Materials

Lovastatin lactone and simvastatin lactone were kind gifts from Merck Sharp & Dohme Ltd (Rahway, NJ, U.S.A.). Fluvastatin sodium salt and SDZ-PSC833 was from Novartis Pharma AG (Basel, Switzerland), and pravastatin sodium salt from Bristol-Meyers Squibb (Sankyo; Japan). Atorvastatin acid was isolated from a commercially available drug. Rhodamine 123 was purchased from Molecular Probes (Eugene, OR, U.S.A.). All other chemicals used were of best quality available and were purchased from Merck (Darmstadt, Germany), Sigma or Fluka (Buchs, Switzerland).

Foetal calf serum and all culture medium contents were from Gibco (Paisley, UK), 96-well microtiter plates were from Costar (Cambridge, MA), Optiplate™-96 were from Packard (Zürich, Switzerland) and the Lab-Tek chambered coverglass system from Nunc (Napperville, IL, U.S.A.).

Cell lines

The murine monocytic leukaemia cell lines P388/P and the doxorubicin-resistant subline P388/MDR were obtained from Dr C. Geroni (Pharmacia & Upjohn, Centro Ricerche e Sviluppo, Milano, Italy). Both cell lines were cultured in RPMI glutamax 1640 supplemented with 10% (v v^{-1}) heat-inactivated foetal calf serum, 1 mM sodium pyruvate, 10 mM HEPES buffer, 0.02 mg ml^{-1} asparagine, 1 × minimum essential medium with non-essential amino acids, 1 × minimum essential medium with vitamins, 0.05 mM β-mercaptoethanol, 50 μg ml^{-1} gentamycin. Culture conditions were 5% CO_2/95% air atmosphere at 37°C.

The P388/MDR cells were continuously grown in the presence of 0.25 μg ml^{-1} doxorubicin. One day before each experiment, cells were grown in doxorubicin-free medium.

Isolation of atorvastatin

Twenty-nine Sortis® tablets (corresponding to a total of 8.4 g of atorvastatin acid) were suspended in a mixture of 151 ml methylene chloride : methanol : hexane : acetic acid (50 : 50 : 50 : 1) (v v^{-1} v^{-1} v^{-1}) and stirred for 2 h. The insoluble excipients were filtered off and the solution was evaporated to dryness at 50°C under vacuum, yielding 800 mg evaporation residue. Final purification was achieved with preparative high-pressure liquid chromatography (h.p.l.c.) using a preparative column filled with 100 g silica gel (15 μm, Merck no. 11635) as stationary phase. Of the above mentioned evaporation residue 500 mg were dissolved in 20 ml methylene chloride : methanol (1 : 1) (v v^{-1}), poured onto the column and eluted with methylene chloride : tert.-butylmethylether : methanol (2 : 2 : 1) (v v^{-1} v^{-1}) as mobile phase with a flow rate of 10 ml min^{-1}. After 2 h, the mobile phase was changed to methylene chloride : tert.-butylmethylether : methanol (1 : 1 : 2) (v v^{-1} v^{-1}) and atorvastatin was eluted immediately within 50 min. The solvents were removed at 50°C under vacuum, yielding 301 mg of drug substance with a purity of 98.3 % (h.p.l.c.). The product was finally characterized with mass spectrometry and ^{1}H-NMR-spectroscopy.

Isolation of atorvastatin lactone and atorvastatin methyl ester

Sixty-six mg of atorvastatin drug substance (isolated as described above) dissolved in 6 ml acetonitrile and 100 μl phosphoric acid (85%) were heated for 3 h at 80°C. After cooling down to room temperature the sample was evaporated to dryness and the evaporation residue was again dissolved in 3 ml methylene chloride : methanol (1 : 1) (v v^{-1}). The solution was poured on three preparative thin layer chromatography plates (Merck no. 5715) and the compounds were subsequently eluted with methylene chloride : tert.-butylmethylether : methanol (90 : 5 : 5) (v v^{-1} v^{-1}). Using 254 nm UV-detection, two zones became visible. Atorvastatin lactone was concentrated in zone 1 with rf = 0.60, whereas atorvastatin methyl ester was concentrated in zone 2 with rf = 0.81. After removal of the separated zones from the plates the combined lactone and methyl ester samples were washed with 3 ml hexane and eluted with 3 ml methylene chloride : methanol (1 : 1) (v v^{-1} v^{-1}). After evaporation to dryness the compounds were lyophilized in 6 ml acetonitrile : water (1 : 1) (v v^{-1}) each, yielding 15.2 mg atorvastatin lactone and 6.9 mg atorvastatin methyl ester. The purity for both compounds was >98% and the structures were confirmed with mass spectrometry and ^{1}H-NMR-spectroscopy.

Preparation of simvastatin acid and lovastatin acid

Simvastatin acid and lovastatin acid were reconstituted as described earlier (Tsuji *et al*., 1993), respectively from simvastatin and lovastatin by hydrolysis in 0.05 N NaOH solution with stirring at 20°C for 30 min. The hydrolysed solutions were adjusted to pH 7.4 with 0.2 N HCl and stored at 4°C until used.

Stock solutions

Stock solutions of test compounds were prepared using dimethyl sulfoxide (DMSO). The rhodamine 123 stock solution was prepared in ethanol (99.8%).

Qualitative HPLC method

Stability in Hank's balanced salt solution (HBSS) was tested for all statins using h.p.l.c. Samples were analysed by chromatography on a heated (60°C) RP18 endecapped Superspher® 100 (Lichrochart® 125-4) column, with isocratic elution and a water : acetonitrile : tert.-butylmethylether:phosphoric acid (85%) (600 : 300 : 70 : 0.2) (v v^{-1} v^{-1} v^{-1}) mobile phase with a constant flow rate of 1 ml min^{-1}. The effluent was monitored using 236 nm UV-detection and diode array.

Fluorimetry

Uptake assay In order to measure net R123 uptake, 2×10^6 cells ml^{-1} were pre-incubated at 37°C for 10 min in the presence or absence of test drugs, followed by incubation with 10 μM R123. After 10 min, uptake was stopped by transferring samples on ice, cells were washed twice in the presence or absence of test drugs at 4°C and lysed in 0.8% Triton X-100, aliquots were transferred into Optiplate™-96 plates and fluorescence of the lysate was analysed with a HTS 7000 Plus Bio Assay Reader (Perkin Elmer Ltd., Buckinghamshire, UK) with 485 nm excitation and 535 nm emission filters.

Efflux assay To load R123 into P388/MDR cells prior to efflux, cells were incubated at 37°C in 1 μM R123 in the presence of 100 μM verapamil in order to block P-gp. After 10 min, incubation was stopped by transferring samples on ice; cells were dispersed into a 96-well microtiter plate (Costar 3799) and washed twice with or without test drugs at 4°C. Efflux was initiated by resuspending the cell pellet in R123-free HBSS with or without test drug (efflux buffer) at room temperature. After 6 min efflux, 200 μl samples of the efflux buffer were analysed for R123 fluorescence in the HTS 7000 Plus Bio Assay Reader (Perkin Elmer Ltd., Buckinghamshire, U.K.) with 485 nm excitation and 535 nm emission filters.

Laser scanning microscopy

The Lab-Tek chambered coverglass containing 0.5×10^6 living cells ml^{-1} in HBSS, supplemented with 1 mM pyruvate for energy supply, was mounted on the stage of a Zeiss LSM 510 inverted laser scanning microscope. An Ar 488 nm laser line and 505–530 nm band-pass filter were used for R123 detection, whereas for the detection of propidium iodide (PI) a 650 nm long-pass filter was used. Objectives used were a Zeiss Plan-Neofluar 63 × oil immersion objective with a numerical aperture of 1.25. Optical sections of 1.1 μm thickness were scanned through the z-plane of the sample.

Autofluorescence of the cells was not detected. After a 10 min incubation phase in 0.15 μM R123 in HBSS at room temperature, test drug was added and time-dependent R123 accumulation in the cells was recorded. Dead cells were stained with PI. Final DMSO and ethanol concentrations were below 0.6 and 1%, respectively. For data analysis, twenty cells in one z-section of each experiment were randomly chosen, with exclusion of death cells, and the pixel intensity was measured using the public domain NIH Image 1.61 program. Data are reported as an average measured pixel intensity.

For kinetic analysis, the R123 accumulation was measured 10 min after addition of statins and 30 min after addition of verapamil and SDZ-PSC833.

Statistical analysis

For estimation of the kinetic parameters, a non-linear regression program was used (Microcal Origin, version 6.0, Origin Microcal Software, Inc., Northampton, MA, U.S.A.). Data are given as means ± s.e.mean. Comparison tests were performed by ANOVA with Bonferroni's adjustment for *post hoc* pairwise comparisons.

Calculation of kinetic parameters

IC_{50}, or the concentration of test compound that exerts a half-maximal inhibition of the R123 transport was calculated from a saturable relationship. R123 concentrations used were 1 μM for the microtiter plate based fluorimetric assay and 0.15 μM for the confocal microscopy based assay.

The inhibitory potency (IP_x), or the capacity of a test compound, at concentration x μM, to inhibit the R123 transport was systematically related to the IP_{100} and IP_{10} of verapamil in the same assay for fluorimetric and confocal assays, respectively.

The resulting IP_{rel} was calculated with formula 1.

$$IP_{rel-x} = \Delta F_{\text{test compound at conc. x}} / \Delta F_{\text{verapamil}}, \qquad (1)$$

where ΔF is the difference in R123 fluorescence in absence and presence of P-gp modulator.

Results

Method validation

In order to characterize P-glycoprotein (P-gp) mediated transport of rhodamine 123 (R123) in our cell system, parental (P388/P) and P-gp over-expressing (P388/MDR) cells were compared for their R123 specific fluorescence levels after incubation in 10 μM R123. The absence of P-gp expression in P388/P cells and the high amount in P388/MDR cells were demonstrated by Western blot earlier in our laboratory (Drewe *et al*., 1999). Figure 1 shows the influence of a representative P-gp inhibitor (verapamil) and energy depletion on the R123 accumulation in P388/MDR cells. In the absence of P-gp modulator, the P388/MDR cells

displayed low fluorescence (26.0%) in comparison to their P388/P cell controls. R123 accumulation up to 75.6% and 62.0% of P388/P cell controls was obtained by P-gp inhibition using 100 μM verapamil and ATP-depletion with NaN_3, NaF respectively. This showed an energy-dependent, P-gp mediated active extrusion of the rhodamine dye in P388/MDR cells.

To analyse a test substance for its P-gp modulating properties, we designed two complementary assays. A microtiter plate based fluorimetric efflux assay with moderate throughput and a more sensitive confocal microscopy based uptake assay with live monitoring of single cells.

The inter-assay variability was normalized by the inhibitory potency of verapamil. For the microtiter plate based assay the coefficients of variation for intra- and inter-assay variability were 3.7 and 9.5% respectively, the confocal microscopy based assay had coefficients of variation of 6.0 and 30.0% in between assays and in between cells respectively.

In order to compare the two above described methods, verapamil and SDZ–PSC833, well-characterized P-gp inhibitors (Pourtier-Manzanedo *et al*., 1992), were analysed. Figure 2 shows the concentration-dependent inhibition of R123 efflux in P388/MDR cells, pre-incubated with 1 μM R123, by SDZ–PSC833 and verapamil. IC_{50} values were 2.7 μM and 6.5 μM respectively.

The concentration-dependent R123 accumulation in P388/MDR cells by SDZ–PSC833 and verapamil were also assessed by confocal microscopy and resulted in IC_{50} values of 0.4 μM and 1.3 μM respectively (Figure 3).

In both methods, SDZ–PSC833 was the more potent inhibitor of P-gp, but IC_{50} values calculated from confocal analysis were 6–5-times lower for SDZ–PSC833 and verapamil, respectively, and a 12% higher difference of the inhibitory potencies was measured, when compared to the fluorimetric assay.

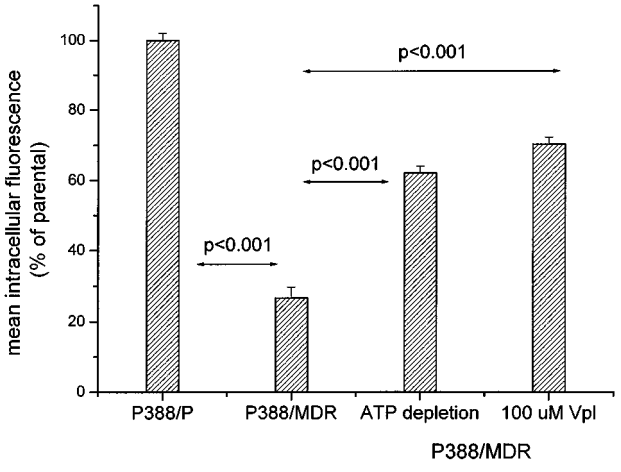

Figure 1 R123 uptake in P388/P and P388/MDR cells and influence of ATP depletion; (NaN_3 10 mM, NaF 10 mM) and a representative P-gp inhibitor (verapamil 100 μM) on R123; restoration in P388/MDR cells. Data are given as percentage of R123 fluorescence in P388/P cells. Values represent means ± s.e.mean for $n=5$, *P*-values are determined by ANOVA with Bonferroni's adjustment for *post hoc* pairwise comparisons.

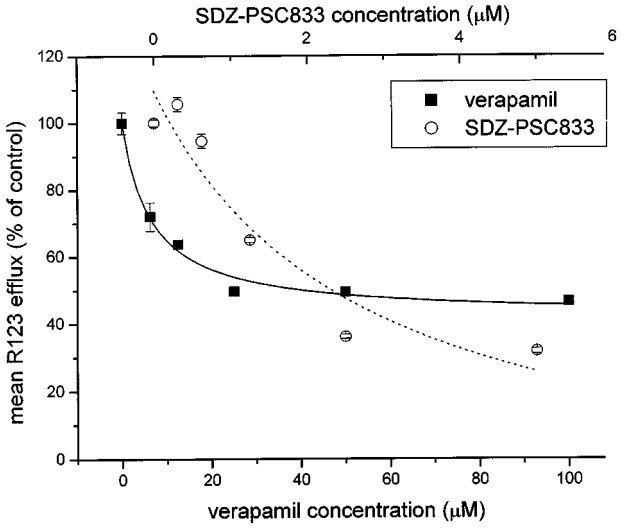

Figure 2 Microtiter plate based fluorimetric assay. Effect of SDZ-PSC833 and verapamil on R123 efflux out of P388/MDR cells, cells were preloaded in 1 μM R123. Data are expressed as percentage of R123 efflux in absence of inhibitor. Values represent means ± s.e.-mean for $n=5$.

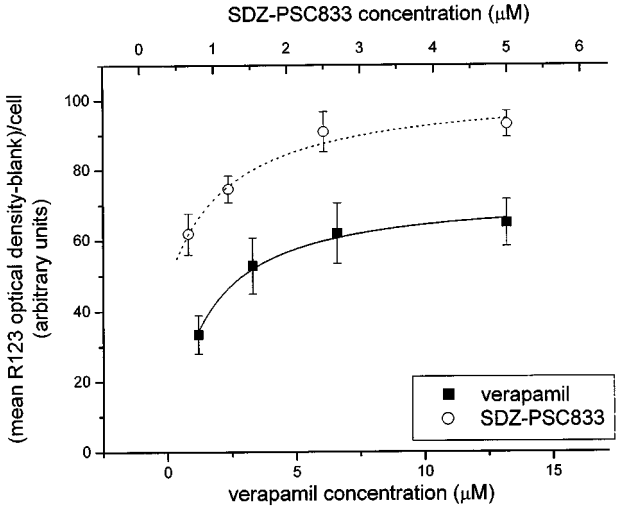

Figure 3 Confocal microscopy based assay. Concentration dependent effect of SDZ-PSC833 and verapamil on intracellular R123 accumulation in P388/MDR cells, pre-incubated with 0.15 μM R123. Data are expressed as blank corrected mean optical densities per cell. Values represent mean ± s.e.mean for 20 cells.

Applications

To study the P-gp modulatory effect of a series of HMG-CoA reductase inhibitors (e.g. atorvastatin, fluvastatin, pravastatin, lovastatin and simvastatin), a combination of both assays was used.

Prior to P-gp affinity studies, possible quenching of R123 fluorescence induced by the test compound was determined by analysing the decrease in fluorescence of a fixed R123 concentration (25 nM) upon addition of increasing concentrations of test compound dissolved in HBSS. Only fluvastatin showed a significant quenching and could reduce the R123 fluorescence up to 55% of its control value.

Five to six concentrations of statins were tested in $n=5$ replicates starting always with the control (0 μM) and 3 μM as the lowest concentration up to at maximum 200 μM of test substance. This range was supposed to cover pharmacological concentrations and never achieved toxic concentrations in the assays. The latter was verified by measuring lactate dehydrogenase activity of the medium and by PI staining (data not shown). None of the drugs showed signs of cytotoxicity above 50 μM concentrations except simvastatin lactone and lovastatin lactone.

Microtiter plate based fluorimetric assay

The microtiter plate based fluorimetric efflux assay (Figure 4 and Table 1), showed that atorvastatin lactone, methyl ester and acid, simvastatin lactone and lovastatin lactone induced a concentration-dependent inhibition of the R123 efflux but not simvastatin acid neither lovastatin acid nor pravastatin did show any significant modulating effect ($P>0.05$). The mentioned quenching confounded fluvastatin efflux data and therefore interpretation of the corrected values was difficult. Estimates of the kinetic parameters resulted in similar values for atorvastatin acid, simvastatin and lovastatin lactone with IC_{50} values of 30.1, 38.2 and 45.8 μM respectively, whereas atorvastatin lactone and methyl ester were more potent inhibitors ($IC_{50}=5.2$ μM and 3.2 μM respectively). Spontaneous and fast lactone hydrolysis in aqueous medium led to variability in efflux data for low simvastatin lactone and lovastatin lactone concentrations. Probably due to interference with the more hydrophilic acid form which does not show P-gp affinity. H.p.l.c. analysis of simvastatin and lovastatin hydrolysis products showed that in aqueous medium, 30% of lactone was transformed to the acid form within 2 h (data not shown), this was consistent with previous reported data (Masters *et al*., 1995).

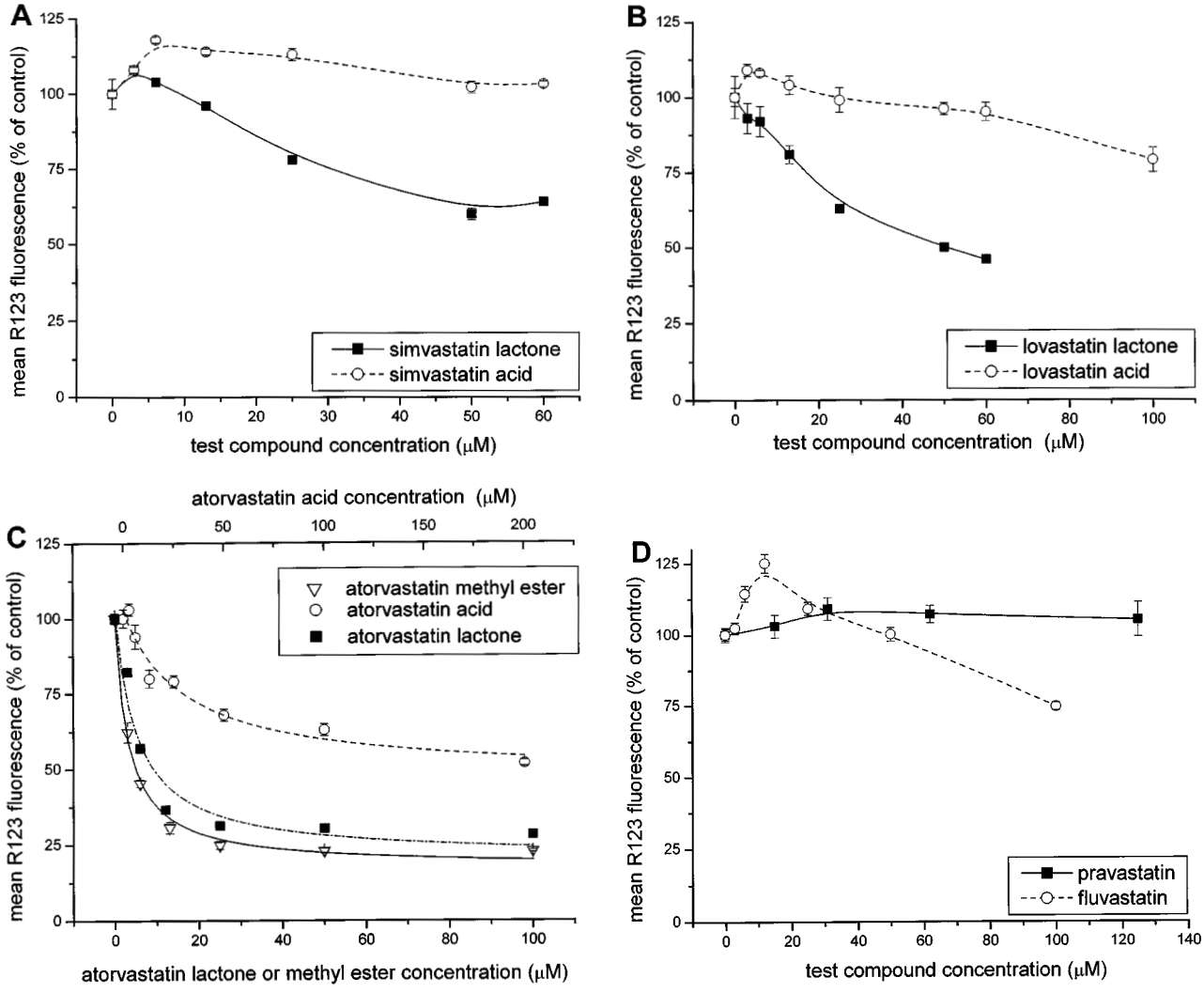

Figure 4 Microtiter plate based fluorimetric assay. Concentration dependent effect of P-gp modulators on R123 efflux out of P388/MDR cells, cells were preloaded in 1 μM R123. (A) Simvastatin lactone and simvastatin acid; (B) lovastatin lactone and lovastatin acid; (C) atorvastatin lactone, atorvastatin methyl ester and atorvastatin acid; (D) pravastatin and fluvastatin (fluvastatin values were corrected for quenching). Data are expressed as percentage of R123 efflux in absence of inhibitor. Values represent means$\pm$s.e.mean for $n=5$.

Table 1 Ranking of a series of P-gp modulators according to their capacity to modulate R123 transport. Comparison of a microtiter plate based fluorimetric efflux assay with a confocal microscopy based accumulation assay and relation to lipophilicity expressed as log *P* values

	ATO methyl ester	*ATO lactone*	*ATO acid*	*SIM lactone*	*SIM acid*	*LOV lactone*	*LOV acid*	*PRA*	*FLU*	*PSC833*	*Verapamil*
Microtiter based fluorimetric assay											
IC_{50}[a] (μM)	3.2±0.4	5.2±0.7	30.1±12.1	38.2±24.8	ND	45.8±14.7	ND	ND	ND	2.7±2.5	6.5±1.5
IP_{rel}[b] (% of $IP_{verapamil}$)	110.3±1.1	110.0±2.0	53.1±3.5	76.9±3.8	−2.9±2.8	90.7±1.8	5.8±3.4	−11.6±5.4	17.6±3.1	127.7±1.5	100±2.2
Confocal microscopy based assay											
IC_{50}[a] (μM)	1.8±1.0	3.1±0.4	21.5±2.2	24.5±67.6	ND	22.54±7.1	ND	ND	ND	0.4±0.04	1.3±0.1
t_{50}[c] (min)	21.9	5.2	27.1	5.3	ND	13.4	ND	ND	ND	29.3	9.6
Compound properties											
Log P	6.64[d] (clog P)	6.04[d] (clog P)	4.06[e]	4.7[f]	2.1[f]	4.3[f]	1.7[f]	−0.23[f]	3.80[d] 3.24[e] (clog P)	ND	3.79[g]

[a]Concentration of test compound needed to reach half-maximal P-gp inhibition. R123 concentration was 1 μM in the microtiter plate based assay and 0.15 μMin the confocal microscopy based assay. Values are calculated with a saturable relationship. [b]Inhibitory potency, calculated with formula (1) using experimental values for R123 efflux, at test compound and verapamil concentrations 50 μM (5 μM for PSC833) and 100 μM respectively. [c]Time needed to reach half-maximal R123 restoration after addition of test compound. [d]SRC, KowWin public domain; [e](Corsini *et al*., 1999), [f](Lennernäs & Fager, 1997), [g]SRC Physprop Database, public domain; ND = not determined.

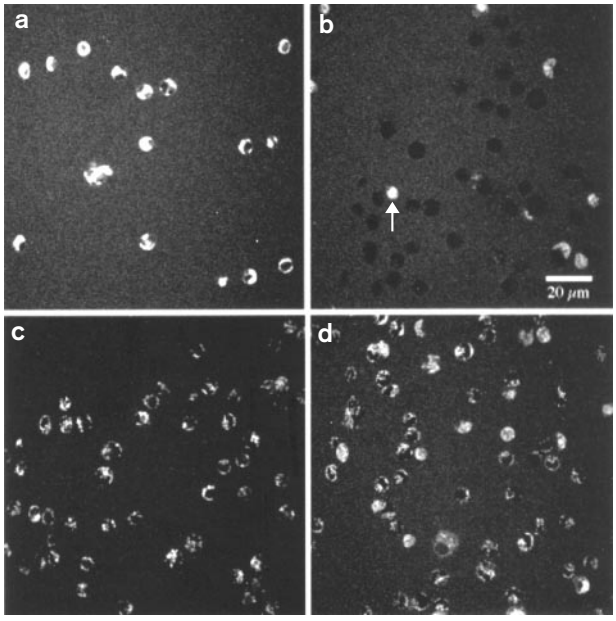

Figure 5 Representative confocal microscopy pictures of P388/P (a), P388/MDR (b) cells after incubation in 0.15 μM R123 and P388/MDR after subsequent addition of verapamil 10 μM (c) and atorvastatin methyl ester 10 μM (d). Addition of test compounds that did not inhibit P-gp, resulted in pictures as shown in (b). R123 stains mitochondria in living cells. Dead cells (pointed by the arrow) show a delocalized cytosolic dye staining. Bar = 20 μM.

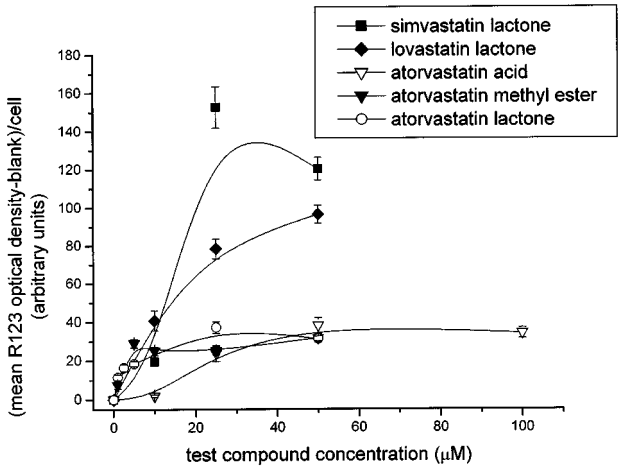

Figure 6 Confocal microscopy based assay. Concentration dependent effect of simvastatin lactone, lovastatin lactone, atorvastatin acid, atorvastatin methyl ester, atorvastatin lactone on intracellular R123 restoration in P388/MDR cells, pre-incubated with 0.15 μM R123. Data are expressed as blank corrected mean optical densities per cell. Values represent mean±s.e.mean for 20 cells.

Confocal microscopy based assay

The confocal microscope based assay was applied in parallel to the above-described method, whereby the statins were analysed for their capacity to induce a R123 accumulation in P388/MDR cells.

Figure 5 shows representative confocal micrographs of P388/P and P388/MDR cells after 10 min incubation in R123 0.15 μM and subsequent treatment with test compounds.

P388/P cells accumulated within minutes a high amount of R123 that is preferentially staining mitochondria. In contrast, the P388/MDR cells did not accumulate the dye for at least 2 h. When cells were naturally energy depleted, a small fraction of the dye could enter the cytosol; adding sodium pyruvate could reverse this reaction. Upon addition of P-gp inhibitors, P388/MDR cells started accumulating R123 resulting in an intracellular staining.

Results obtained by quantitative analysis of intracellular R123 fluorescence upon addition of different concentrations of test compound are represented in Figure 6 and are

consistent with data provided by the microtiter plate based fluorimetric assay. The procedure of image capture and subsequent quantification of intracellular dye fluorescence with the analysis program NIH Image has been validated before (Miller & Pritchard, 1991). The observed ranking of P-gp modulators based on apparent IC_{50} was atorvastatin methyl ester $\geqslant$ atorvastatin lactone $>$ lovastatin lactone $\cong$ simvastatin lactone $\cong$ atorvastin acid (Table 1). No significant ($P>0.05$) inhibitory effect was seen for pravastatin neither fluvastatin, nor for simvastatin and lovastatin in their acid forms.

The time needed to reach half-maximal R123 accumulation (t_{50}) was calculated with a saturable relationship and resulted in a higher accumulation rate for simvastatin lactone, atorvastatin lactone and lovastatin lactone than for atorvastatin methyl ester and atorvastatin acid (Table 1).

Discussion

The aim of the present work was to study P-gp modulation by a series of HMG-CoA reductase inhibitors. Recently, atorvastatin has been identified as P-gp substrate (Boyd *et al*., 2000; Wu *et al*., 2000), for some of the other statins P-gp interaction was assumed but not yet proven (Christians *et al*., 1998; Dimitroulakos & Yeger, 1996; Lindahl *et al*., 1999).

The high hepatic extraction (Corsini *et al*., 1999; Lennernäs & Fager, 1997) of these compounds gives evidence for transporter-mediated excretion into the bile by P-gp and MRP2 (cMOAT), which are located at the canalicular plasma membrane (Müller & Jansen, 1997).

Because of its hydrophilicity, pravastatin's passive diffusion into hepatocytes is limited and transport across the sinusoidal membrane is facilitated by the sodium dependent organic anion transporter, hOATP2; this process can be inhibited by atorvastatin, simvastatin and lovastatin, suggesting that these statins possibly compete for this transport (Hsiang *et al*., 1999). Bile excretion of pravastatin is facilitated by MRP2 (Yamazaki *et al*., 1997).

When drugs compete for a common transporter, drug interactions are likely to occur. Until now, effects on metabolism explained most clinical interactions with HMG-CoA reductase inhibitors. Pravastatin is not metabolized by the CYP450 3A4 system, therefore it is likely that the increased pravastatin plasma concentration after co-administration with cyclosporin A is caused by interaction on one or more transporter proteins.

We established two *in vitro* assay systems that can be used complementary to assess drug mediated P-gp modulation. The confocal microscopy based assay can be used as a first-line highly sensitive method to analyse new substances for their effect on R123 accumulation properties. The need for only small amounts of compounds and cells and the real-time process monitoring makes it an appropriate method for early preclinical drug screening. Potential cellular toxicity and changes in the dye accumulation pattern is readily detectable. The microtiter plate based fluorimetric assay performs a fast further screening of extended concentrations and saturation kinetics. The latter assay allows a higher throughput of samples.

The differences in IC_{50} values between the two assays (Table 1) were most likely under-estimations of the inhibitory effect measured by the fluorimetric based assay. They could be explained by the different experimental designs, efflux versus uptake assays. During the efflux phase after loading of the cells, the direction of R123 passive diffusion and P-gp mediated extrusion was the same and sink conditions were maintained. Whereas in the uptake assay the two fluxes were in opposite directions. The same observations were reported in flow cytometry assays (Wang *et al*., 2000). Also an intracellular trapping of dye had to be considered, a fraction that would not be available anymore for active extrusion. Furthermore, efflux was stopped after 5 min, where uptake was analysed at least for 10–30 min after addition of modulator. In addition, a 6.7-times lower R123 concentration was used in confocal microscopy.

In confocal microscopy, calculated IC_{50} values for the statins may be over-estimated, due to the small number of samples in the low concentration range of the dose-response curve. The latter method showed an unexpectedly high IP_{rel-50} value for simvastatin (268%) and lovastatin (215%) in their lactone forms that can not be explained yet. Nevertheless, the overall ranking from strong over weak to none P-gp inhibition and affinity was: SDZ-PSC833 $>$ atorvastatin methyl ester $\geqslant$ atorvastatin lactone $>$ verapamil $>$ simvastatin lactone $\cong$ lovastatin lactone $\cong$ atorvastatin acid $>$ pravastatin $\cong$ fluvastatin. This ranking was comparable for both methods and consistent with results of other groups for known inhibitors (Pourtier-Manzanedo *et al*., 1992).

Lipophilicity

In previous structure–activity–relationship studies on modulators of P-glycoprotein (Ecker & Chiba, 1995; Khan *et al*., 1998; Zamora *et al*., 1988), it has been shown that a correlation exists between the log *P* values and the chemosensitizing activity of a compound. In the present study, high affinity (low IC_{50}) correlates with rather high log *P* values (Table 1) with the exception of fluvastatin, which has log *P* higher than 3. Our findings suggest that fluvastatin does not interact with P-gp, but the exact mechanism of interaction with cell membranes has to be elucidated.

Its ability to decrease the surface tension at the membrane surface and postulated interaction with lipids that could result in an altered membrane fluidity has been reported (Lindahl *et al*., 1999). Involvement of other transporters such as cMOAT and OATP, both anion transporters, cannot be excluded and will be investigated in future experiments.

Looking at the R123 accumulation rate initiated by a modulator, fast and slow processes can be distinguished. In addition, solubility of the modulator, transport rate through membrane bilayer and affinity for the transporter can influence P-gp interaction. The reported lower trans-membrane movement rate for SDZ-PSC833 compared to the one for verapamil (Ambudkar *et al*., 1999; Eytan & Kuchel, 1999; Smith *et al*., 1998), is well reflected in its high t_{50} value of 29.3 min in comparison to that of verapamil ($t_{50}=9.6$ min). Furthermore, the fast onset of P-gp inhibition initiated by verapamil was proven in intestinal perfusion studies with talinolol in healthy volunteers (Grammaté & Oertel, 1999).

Our data suggested that the more lipophilic HMG-CoA reductase inhibitors had a higher R123 uptake rate (lower t_{50}) (Table 1).

Table 2 Reported drug interactions with HMG-CoA reductase inhibitors

Compound	*Interaction site*	*Manifestation*	*Atorvastatin*	*Simvastatin*	*Lovastatin*	*Pravastatin*	*Fluvastatin*
Digoxin	P-gp	Fold ↑ $AUC_{digoxin}$	15%[a]	+0.3 ng/ml[f]	?[f]	NS[f]	NS; renal Cl of digoxin↑[b]
Cyclosporin A	P-gp + CYP3A4	Fold ↑ AUC_{statin}	Rhabdomyolysis[c]	6[d]	20[e]	5-23[e]	1.9[e]
Diltiazem	P-gp + CYP3A4	Fold ↑ plasma $concentration_{statin}$	NR	3.6 (lactone) 3.7 (acid)[h]	4.3[h]	NS[h]	NR
Itraconazole	P-gp + CYP3A4	Fold ↑ AUC_{statin}	2[l]	20[l]	15[l]	NS[l]	NS[l]
Mibefradil	P-gp + CYP3A4	Fold ↑ AUC_{statin}	3.4[j]	7[k]	NR	1.2[j]	1.1[k]
		IC_{50} of mibefradil (μM) for inhibition of statin metabolism (*in vitro*)	0.4[i]	0.6[i]	0.35[i]	NR[i]	NS[i]
Gemfibrozil	CYP3A4		Rhabdomyolysis[f]	Rhabdomyolysis[f]	Rhabdomyolysis, creatine kinase 20-30x↑[f]	NS ↑ AUC of metabolites[f]	NS[f]

AUC = area under the plasma concentration – time curve; NR = not reported; NS = no significant change; ? = no clinically significant interaction, information on plasma concentration not given. [a](Boyd *et al*., 2000), [b](Deslypere, 1994), [c](Maltz *et al*., 1999), [d](Lennernäs & Fager, 1997), [e](Corsini *et al*., 1999), [f](Stockley, 1999), [g](Mousa *et al*., 2000), [h](Azie *et al*., 1998), [i](Prueksaritanont *et al*., 1999), [j](Donahue *et al*., 1999), [k](Mück, 2000), [l](Bottorff, 1999).

Drug – drug interactions

What is the value of this information to predict potential pharmacokinetic drug interactions? Recently, it was demonstrated that mibefradil is an inhibitor of CYP450 3A4 (Prueksaritanont *et al*., 1999). Mibefradil is also shown to be a P-gp inhibitor (Wandel *et al*., 2000).

Itraconazole, a P-gp inhibitor and CYP450 3A4 substrate, has a clinical interaction pattern with statins, that could be explained by both P-gp and CYP450 3A4 effects. Among the reported drug interactions (Table 2), the increase in digoxin (a P-gp substrate which is not metabolized by CYP450 3A4) plasma levels under concomitant administration of atorvastatin and simvastatin can be explained by P-gp inhibition alone.

Increased statin levels after co-administration with cyclosporin A can be explained both by P-gp and CYP450 3A4 inhibition. However the 5 – 23-fold increased pravastatin bioavailability is more likely caused by cMOAT inhibition rather than an effect on P-gp and CYP450 3A4. In addition, statins that showed P-gp modulating properties in our assays, are presumably also transported by the latter transporter.

Our *in vitro* cell system can be used to screen potential drug interactions, but well-designed clinical studies will still be needed to prove the hypothesis. A variety of other factors such as genetic polymorphism and interindividual variation (Hoffmeyer *et al*., 2000; Lown *et al*., 1997) or susceptibility to adverse effects are further determinants of clinical outcome.

In conclusion, the present study demonstrated that the described methods are able to analyse P-gp modulating properties of test compounds *in vitro*. We have shown that atorvastatin acid, atorvastatin methyl ester and lactone, simvastatin lactone and lovastatin lactone are P-gp modulators. No effect was seen with simvastatin acid, lovastatin acid, fluvastatin and pravastatin. Clinical and *in vitro* data suggest that P-gp modulating statins have a general trend to high probability for drug interactions.

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References

AMBUDKAR, S.V., DEY, S., HRYCYNA, C.A., RAMACHANDRA, M., PASTAN, I. & GOTTESMAN, M.M. (1999). Biochemical, cellular, and pharmacological aspects of the multidrug transporter. *Annu. Rev. Pharmacol. Toxicol.,* **39,** 361 – 398.

AZIE, N.E., BRATER, D.C., BECKER, P.A., JONES, D.R. & HALL, S.D. (1998). The interaction of diltiazem with lovastatin and pravastatin. *Clin. Pharmacol. Ther.,* **64,** 369 – 377.

BOESCH, D., GAVERIAUX, C., JACHEZ, B., POURTIER-MANZANEDO, A., BOLLINGER, P. & LOOR, F. (1991). *In vivo* circumvention of P-glycoprotein-mediated multidrug resistance of tumor cells with SDZ PSC 833. *Cancer Res,* **51,** 4226 – 4233.

BOTTORFF, M. (1999). 'Fire and forget?' - pharmacological considerations in coronary care. *Atherosclerosis,* **147**(Suppl 1): S23 – S30.

BOYD, R.A., STERN, R.H., STEWART, B.H., WU, X., REYNER, E.L., ZEGARAC, E.A., RANDINITIS, E.J. & WHITFIELD, L. (2000). Atorvastatin coadministration may increase digoxin concentrations by inhibition of intestinal P-glycoprotein-mediated secretion. *J. Clin. Pharmacol.,* **40,** 91 – 98.

CHENG, H., SCHWARTZ, M.S., VICKERS, S., GILBERT, J.D., AMIN, R.D., DEPUY, B., LIU, L., ROGERS, J.D., POND, S.M. & DUNCAN, C.A. (1994). Metabolic disposition of simvastatin in patients with T-tube drainage. *Drug Metab. Dispos.,* **22,** 139 – 142.

CHRISTIANS, U., JACOBSEN, W. & FLOREN, L.C. (1998). Metabolism and drug interactions of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors in transplant patients: are the statins mechanistically similar?. *Pharmacol. Ther.,* **80,** 1 – 34.

CORSINI, A., BELLOSTA, S., BAETTA, R., FUMAGALLI, R., PAOLETTI, R. & BERNINI, F. (1999). New insights into the pharmacodynamic and pharmacokinetic properties of statins. *Pharmacol. Ther.,* **84,** 413–428.

DESLYPERE, J.P. (1994). Clinical implications of the biopharmaceutical properties of fluvastatin. *Am. J. Cardiol.,* **73,** 12D–17D.

DIMITROULAKOS, J. & YEGER, H. (1996). HMG-CoA reductase mediates the biological effects of retinoic acid on human neuroblastoma cells: lovastatin specifically targets P- glycoprotein-expressing cells. *Nat. Med.,* **2,** 326–333.

DONAHUE, S., LACHMAN, L., NORTON, J., FRANC, J. & CHEN, L. (1999). Mibefradil significantly increases serum concentrations of atorvastatin but not pravastatin. *Clin. Pharmacol. Ther.,* **65,** 179.

DREWE, J., GUTMANN, H., FRICKER, G., TÖRÖK, M., BEGLINGER, C. & HUWYLER, J. (1999). HIV protease inhibitor ritonavir: a more potent inhibitor of P-glycoprotein than the cyclosporine analog SDZ PSC 833. *Biochem. Pharmacol.,* **57,** 1147–1152.

ECKER, G. & CHIBA, P. (1995). Structure-activity-relationship studies on modulators of the multidrug transporter P-glycoprotein – an overview. *Wien. Klin. Wochenschr.,* **107,** 681–686.

EYTAN, G.D. & KUCHEL, P.W. (1999). Mechanism of action of P-glycoprotein in relation to passive membrane permeation. *Int. Rev. Cytol.,* **190,** 175–250.

FRICKER, G., GUTMANN, H., DROULLE, A., DREWE, J. & MILLER, D.S. (1999). Epithelial transport of anthelmintic ivermectin in a novel model of isolated proximal kidney tubules. *Pharm. Res.,* **16,** 1570–1575.

GRAMMATÉ, T. & OERTEL, R. (1999). Intestinal secretion of intravenous talinolol is inhibited by luminal R-verapamil. *Clin. Pharmacol. Ther.,* **66,** 239–245.

HALPIN, R.A., ULM, E.H., TILL, A.E., KARI, P.H., VYAS, K.P., HUNNINGHAKE, D.B. & DUGGAN, D.E. (1993). Biotransformation of lovastatin. V. Species differences in in vivo metabolite profiles of mouse, rat, dog, and human. *Drug. Metab. Dispos.,* **21,** 1003–1011.

HOFFMEYER, S., BURK, O., VON RICHTER, O., ARNOLD, H.P., BROCKMOLLER, J., JOHNE, A., CASCORBI, I., GERLOFF, T., ROOTS, I., EICHELBAUM, M. & BRINKMANN, U. (2000). Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity in vivo. *Proc. Natl. Acad. Sci. USA.,* **97,** 3473–3478.

HSIANG, B., ZHU, Y., WANG, Z., WU, Y., SASSEVILLE, V., YANG, W.P. & KIRCHGESSNER, T.G. (1999). A novel human hepatic organic anion transporting polypeptide (OATP2). Identification of a liver-specific human organic anion transporting polypeptide and identification of rat and human hydroxymethylglutaryl- CoA reductase inhibitor transporters. *J. Biol. Chem.,* **274,** 37161–37168.

HUNTER, J. & HIRST, B.H. (1997). Intestinal secretion of drugs: The role of P-glycoprotein and related drug efflux systems in limiting oral drug absorption. *Adv. Drug. Delivery. Rev.,* **25,** 129–157.

KHAN, E.U., REICHEL, A., BEGLEY, D.J., ROFFEY, S.J., JEZEQUEL, S.G. & ABBOTT, N.J. (1998). The effect of drug lipophilicity on P-glycoprotein-mediated colchicine efflux at the blood-brain barrier. *Int. J. Clin. Pharmacol. Ther.,* **36,** 84–86.

KIM, R.B., WANDEL, C., LEAKE, B., CVETKOVIC, M., FROMM, M.F., DEMPSEY, P.J., RODEN, M.M., BELAS, F., CHAUDHARY, A.K., RODEN, D.M., WOOD, A.J. & WILKINSON, G.R. (1999). Interrelationship between substrates and inhibitors of human CYP3A and P-glycoprotein. *Pharm. Res.,* **16,** 408–414.

LENNERNÄS, H. & FAGER, G. (1997). Pharmacodynamics and pharmacokinetics of the HMG-CoA reductase inhibitors. Similarities and differences. *Clin. Pharmacokinet.,* **32,** 403–425.

LINDAHL, A., PERSSON, B., UNGELL, A.L. & LENNERNAS, H. (1999). Surface activity and concentration dependent intestinal permeability in the rat. *Pharm. Res.,* **16,** 97–102.

LINDAHL, A., SANDSTROM, R., UNGELL, A.L., ABRAHAMSSON, B., KNUTSON, T.W., KNUTSON, L. & LENNERNAS, H. (1996). Jejunal permeability and hepatic extraction of fluvastatin in humans. *Clin. Pharmacol. Ther.,* **60,** 493–503.

LOWN, K.S., MAYO, R.R., LEICHTMAN, A.B., HSIAO, H.L., TURGEON, D.K., SCHMIEDLIN-REN, P., BROWN, M.B., GUO, W., ROSSI, S.J., BENET, L.Z. & WATKINS, P.B. (1997). Role of intestinal P-glycoprotein (mdr1) in interpatient variation in the oral bioavailability of cyclosporine. *Clin. Pharmacol. Ther.,* **62,** 248–260.

MALTZ, H.C., BALOG, D.L. & CHEIGH, J.S. (1999). Rhabdomyolysis associated with concomitant use of atorvastatin and cyclosporine. *Ann. Pharmacother.,* **33,** 1176–1179.

MASTERS, B.A., PALMOSKI, M.J., FLINT, O.P., GREGG, R.E., WANGIVERSON, D. & DURHAM, S.K. (1995). In vitro myotoxicity of the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, pravastatin, lovastatin, and simvastatin, using neonatal rat skeletal myocytes. *Toxicol. Appl. Pharmacol.,* **131,** 163–174.

MILLER, D.S. & PRITCHARD, J.B. (1991). Indirect coupling of organic anion secretion to sodium in teleost (*Paralichthys lethostigma*) renal tubules. *Am. J. Physiol.,* **261,** R1470–R1477.

MOUSA, O., BRATER, D.C., SUNBLAD, K.J. & HALL, S.D. (2000). The interaction of diltiazem with simvastatin. *Clin. Pharmacol. Ther.,* **67,** 267–274.

MÜCK, W. (2000). Metabolic interactions between mibefradil and HMG-CoA reductase inhibitors: linking in vitro with in vivo information. *Br. J. Clin. Pharmacol.,* **49,** 87–90.

MÜLLER, M. & JANSEN, P.L. (1997). Molecular aspects of hepatobiliary transport. *Am. J. Physiol.,* **272,** G1285–G1303.

POURTIER-MANZANEDO, A., DIDIER, A.D., MULLER, C.D. & LOOR, F. (1992). SDZ PSC 833 and SDZ 280–446 are the most active of various resistance-modifying agents in restoring rhodamine-123 retention within multidrug resistant P388 cells. *Anticancer. Drugs.,* **3,** 419–425.

PRUEKSARITANONT, T., MA, B., TANG, C., MENG, Y., ASSANG, C., LU, P., REIDER, P.J., LIN, J.H. & BAILLIE, T.A. (1999). Metabolic interactions between mibefradil and HMG-CoA reductase inhibitors: an in vitro investigation with human liver preparations. *Br. J. Clin. Pharmacol.,* **47,** 291–298.

SCHINKEL, A.H. (1997). The physiological function of drug-transporting P-glycoproteins. *Semin. Cancer. Biol.,* **8,** 161–170.

SHAPIRO, A.B., CORDER, A.B. & LING, V. (1997). P-glycoprotein-mediated Hoechst 33342 transport out of the lipid bilayer. *Eur. J. Biochem.,* **250,** 115–121.

SMITH, A.J., MAYER, U., SCHINKEL, A.H. & BORST, P. (1998). Availability of PSC833, a substrate and inhibitor of P-glycoproteins, in various concentrations of serum. *J. Natl. Cancer. Inst.,* **90,** 1161–1166.

STOCKLEY, I.H. (1999). *Drug interactions, A source book of adverse interactions, their mechanisms, clinical importance and management*. London: Pharmaceutical Press.

TAMAI, I., TAKANAGA, H., MAEDA, H., YABUUCHI, H., SAI, Y., SUZUKI, Y. & TSUJI, A. (1997). Intestinal brush-border membrane transport of monocarboxylic acids mediated by proton-coupled transport and anion antiport mechanisms. *J. Pharm. Pharmacol.,* **49,** 108–112.

TANG, B.K. & KALOW, W. (1995). Variable activation of lovastatin by hydrolytic enzymes in human plasma and liver. *Eur. J. Clin. Pharmacol.,* **47,** 449–451.

TRANSON, C., LEEMANN, T. & DAYER, P. (1996). In vitro comparative inhibition profiles of major human drug metabolising cytochrome P450 isozymes (CYP2C9, CYP2D6 and CYP3A4) by HMG-CoA reductase inhibitors. *Eur. J. Clin. Pharmacol.,* **50,** 209–215.

TSUJI, A., SAHEKI, A., TAMAI, I. & TERASAKI, T. (1993). Transport mechanism of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors at the blood-brain barrier. *J. Pharmacol. Expl. Ther.,* **267,** 1085–1090.

WACHER, V.J., WU, C.Y. & BENET, L.Z. (1995). Overlapping substrate specificities and tissue distribution of cytochrome P450 3A and P-glycoprotein: implications for drug delivery and activity in cancer chemotherapy. *Mol. Carcinog.,* **13,** 129–134.

WANDEL, C., KIM, R.B., GUENGERICH, F.P. & WOOD, A.J. (2000). Mibefradil is a P-glycoprotein substrate and a potent inhibitor of both P-glycoprotein and CYP3A In vitro. *Drug. Metab. Dispos.,* **28,** 895–898.

WANG, E.-J., C.N., C., R.P., C. & JOHNSON, W.W. (2000). In vitro flow cytometry method to quantitatively assess inhibitors of P-glycoprotein. *Drug. Metab. Dispos.,* **28,** 522–528.

WU, X., WHITFIELD, L.R. & STEWART, B.H. (2000). Atorvastatin transport in the Caco-2 cell model: contributions of P-glycoprotein and the proton-monocarboxylic acid co-transporter. *Pharm. Res.,* **17,** 209–215.

YAMAZAKI, M., AKIYAMA, S., NI'INUMA, K., NISHIGAKI, R. & SUGIYAMA, Y. (1997). Biliary excretion of pravastatin in rats: contribution of the excretion pathway mediated by canalicular multispecific organic anion transporter. *Drug. Metab, Dispos.,* **25,** 1123–1129.

YU, D.K. (1999). The contribution of P-glycoprotein to pharmacokinetic drug-drug interactions. *J. Clin. Pharmacol.,* **39,** 1203–1211.

ZAMORA, J.M., PEARCE, H.L. & BECK, W.T. (1988). Physical-chemical properties shared by compounds that modulate multidrug resistance in human leukemic cells. *Mol. Pharmacol.,* **33,** 454–462.

ZHANG, Y., LEONESSA, F., CLARKE, R. & WAINER, I.W. (2000). Development of an immobilized P-glycoprotein stationary phase for online liquid chromatographic determination of drug-binding affinities. *J. Chromatogr. B. Biomed. Sci. Appl.,* **739,** 33–37.

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