HMG-CoA Reductase Inhibitors (Statins) Characterized as Direct Inhibitors of P-Glycoprotein

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Purpose. HMG-CoA reductase inhibitors (statins) are commonly prescribed for lipid lowering to treat hypercholesterolemia. Although they are well tolerated, their pharmacokinetic interactions with other drugs can lead to some adverse clinical consequences. The avenue of interaction has been asserted to be CYP3A4 because most (or all) known interactions are with CYP3A4 inhibitors, and statin oxidative metabolism is mediated by CYP3A4 as well as other CYP enzymes. However, these same drugs that exert a clinical pharmacokinetic effect on statin disposition are generally also P-gp substrates/inhibitors; hence, this transporter may be, or may contribute to, the mechanism of interaction.

Methods. This study shows directly, as well as quantifies, the inhibition of P-gp-mediated transport of a fluorescent marker substrate.

Results. Lovastatin and simvastatin are very potent and effective inhibitors of P-gp transport with IC_{50}'s of 26 and 9 μM, respectively, for the human enzyme. Atorvastatin is also an effective P-gp inhibitor, but at higher concentrations. Uniquely, pravastatin, whose functional groups render it an inferior inhibitor of P-gp in the whole cell, had no effect in this assay. This result is consistent with known clinical interactions. The effect of these statins on ATP consumption by P-gp was also assessed, and the K_m results were congruent with the IC_{50} observations.

Conclusions. Therefore, the clinical interactions of statins with other drugs may be due, in part or all, to inhibition of P-gp transport.

KEY WRODS: p-glycoprotein; statins; MDR; inhibitor; HMG-CoA.

INTRODUCTION

Cellular resistance to a wide spectrum of xenobiotics and endogenous lipophilic amphiphiles has been a subject of increasing interest to those concerned with the disposition of many therapeutic drugs (1). Of the various forms of resistance, the phenomenon of a multidrug resistance (MDR) transporter has received the most attention, as it plays a major role in the disposition of many drugs (2–5). The most extensively studied MDR transporter, the gene product of MDR1, is a ~170 kDa membrane glycoprotein known as P-glycoprotein (P-gp). Other members of this family include the multidrug resistance protein (MRP1, MRP2, MRP3, etc.).

These transport proteins are ATP-driven pumps that remove xenobiotics from the interior of cells. Expression of P-gp in normal human tissues—particularly within the cellular membranes of the gastrointestinal tract, liver, blood-brain barrier, adrenals, and kidneys—suggests that the enzyme plays a role in cellular protection as well as in secretion and/or disposition (2). While the primary function of this protein is unknown, its ability to confer resistance to a wide variety of structurally and chemically unrelated compounds remains impressive. Indeed, the substrate list for this transporter now seems to share a similar tolerance or acceptance as cytochrome P450 3A4 (CYP3A4), the predominant intestinal and hepatic cytochrome P450 oxygenase, and may even prove to be more extensive in its substrate recognition.

As a member of the ATP-binding cassette (ABC) superfamily of transporters, P-gp possesses 2 ATP binding sites and uses ATP (via hydrolysis) as the source of energy for 'translocating' substrates. The substrates enter from the lipid bilayer, and can bind to 2 (or more) nonidentical sites (6,7). Moreover, allosteric and perhaps synergistic effects have been observed for certain substrate combinations and conditions (8).

Increased serum concentrations of low-density lipoprotein cholesterol and total cholesterol is linked to arteriosclerosis, ultimately leading to hypertension, coronary heart disease, and stroke—major causes of death in developed countries. Pravastatin, simvastatin, lovastatin, and atorvastatin are inhibitors of HMG-CoA reductase, the rate limiting step in de novo cholesterol synthesis (9), and are widely used to treat hypercholesterolemia (10). Though generally well tolerated, HMG-CoA reductase inhibitors are associated with various adverse effects (11), most notably hepatotoxicity and acute muscle toxicity. Lovastatin monotherapy has ~0.1% risk of skeletal muscle toxicity that dramatically increases when lovastatin is combined with drugs such as cyclosporine, itraconazole, ketoconazole, or erythromycin (10) and is as high as 30% within 1 year in patients treated with cyclosporine (12). All drugs elevating plasma concentrations of statins and, thus, potentially leading to skeletal muscle toxicity are known CYP3A4 and MDR1 (P-glycoprotein) substrates and/or inhibitors (13). Lovastatin concentration increased more than 20-fold when co-administered with itraconazole (14), and cyclosporine treatment significantly increased lovastatin accumulation (15). Simvastatin oral bioavailability is also significantly increased by treatment with itraconazole or cyclosporin (13,16–18). Moreover, acute interactions have been observed with the administration of both digoxin, asserted to be a P-gp substrate/inhibitor, and simvastatin (19). Pravastatin exposure can increase in the presence of cyclosporine (20) despite a lack of significant interaction with CYP3A4 (16). However, itraconazole, a CYP3A4 and Pgp inhibitor, had no significant effect on the systemic exposure to pravastatin (16). Atorvastatin can clinically interact with ethinyl estradiol and erythromycin (21), both asserted to be inhibitors of CYP3A4 and P-gp. Additionally, coadministration of atorvastatin with digoxin will increase systemic exposure to digoxin (22). Lovastatin-induced cytotoxicity in MDR1-expressing cell lines suggests that it is a substrate/inhibitor of the MDR1 (Pgp) transporter (23). Permeability polarity in Caco-2 cell monolayers of atorvastatin indicate that it is a substrate of Pgp (22,24). Pravastatin, however, seems to be a substrate for MRP2 based on both *in vitro* experiments using canalicular membrane vesicles and *in vivo* using Eisai hyper-

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[3] **ABBREVIATIONS:** P-gp, P-glycoprotein; ABC, ATP-binding cassette; MDR, multidrug resistance; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; DNR, daunorubicin; Rho, rhodamine 123.

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biliruminemic rats (25). Other potential drug interactions worth noting are the consequences of increased expression of the mdr2 and mdr1b gene products with oral administration of pravastatin or simvastatin in rats (26).

The objective of this study was to quantify the interactions of the most prescribed statins upon the ubiquitous MDR1 ABC transporter known as P-glycoprotein. Using two different types of assessment, we show herein the distinct potent interaction by all but pravastatin.

MATERIALS AND METHODS

Chemicals

Lovastatin, simvastatin, atorvastatin, and pravastatin were from Schering-Plough compound resources. Daunorubicin (DNR), verapamil, colchicine, cyclosporin A, mannitol, dithiothreitol, ATP disodium, ammonium molybdate, ascorbic acid, sodium meta-arsenite, aprotinin, leupeptin, EGTA, EDTA, HEPES, ouabain, phenylmethylsulfonyl fluoride, and TRIZMA base were purchased from Sigma Chemical Co. (St. Louis, MO). Hanks' balanced salt solution, Alpha Minimum Essential Medium, DMEM, penicillin/streptomycin, fetal bovine serum (FBS), and trypsin-EDTA were obtained from Life Technologies, Inc. (Rockville, MD). Sodium orthovanadate was purchased from Pfaltz & Bauer Inc. (Waterbury, CT). Microplates (Costar 96-well), plastic tubes, and cell culture flasks (75 cm^2) were purchased from Corning Inc. (Corning, NY). All other reagents were of the highest grade commercially available.

Cell Lines

CR1R12 cell line, provided by Dr. Alan Senior (Univ. of Rochester), was maintained in complete α-minimum essential medium (α-MEM) supplemented with 10% FBS, penicillin/streptomycin (50 units/50 μg/ml) in a 5% CO_2-95% air atmosphere at 37°C. Colchicine (0.5 μg/ml) was added to the culture medium. Cells were grown to 80–90% confluency and treated with trypsin-EDTA before subculturing. The 3T3 G185 cell line presenting the gene product of human MDR1 was licensed from NIH and maintained in DMEM.

FACS Flow Cytometry

Fluorescence measurements of individual cells were performed using a Becton-Dickinson FACScalibur fluorescence-activated cell sorter (San Jose, CA), equipped with an ultraviolet argon laser (excitation at 488 nm, emission at 530/30 and 570/30 nm band-pass filters). Analysis was gated to include single cells on the basis of forward and side light-scatter and was based on acquisition of data from 10,000 cells. Log fluorescence was collected and displayed as single-parameter histograms. A direct functional assay for the P-gp efflux pump in CR1R12 cells was performed with the flow cytometer (27).

Cell Viability Test

Cell viability was assessed using exclusion of 0.4% trypan blue as well as propidium iodide staining. Dead cells in which propidium iodide was bound to double strands of DNA or RNA were detected in certain regions of the cytometry dot plots and not included in the final data calculations.

Calculation of Relative Fluorescence

The DNR fluorescence intensity of individual cells was recorded as histograms. The mean fluorescence intensity of 10,000 cells was used for comparison among different conditions. Vanadate was selected as a positive control to normalize the measurements because it can inactivate the P-gp efflux pump. Relative fluorescence was used for quantitation and comparison among different compounds. The relative fluorescence (% inactivation) represents a ratio obtained through the following formula: the geometric mean fluorescence of a discrete sample divided by the geometric mean fluorescence in the presence of 5 mM vanadate, times 100 or expressed as

$$\text{Relative fluorescence} = \frac{\text{Fluorescence of sample geometric mean}}{\text{Fluorescence of reference std. geometric mean}} \times 100$$

Membrane Microsome Preparations

CR1R12 cell membranes enriched with the MDR1 gene product transport enzyme were used for preparation of membrane microsomes. Cells were washed with complete Hanks' buffer before being resuspended in 10 ml lysis buffer (Tris-HCl, 50 mM; mannitol, 50 mM; EGTA, 2 mM; and dithiothreitol, 2 mM; pH 7.0 at 25°C) containing protease inhibitors (phenylmethylsulfonyl fluoride, 1 mM; aprotinin, 10 μg/ml; leupeptin, 10 μg/ml). All subsequent steps were performed at 4°C. The cells were lysed by nitrogen cavitation (Parr Instrument Co., Moline, IL) at 500 psi for 15 min twice. Nuclei and mitochondria were sedimented by centrifugation at 4000 × *g* for 10 min. The microsomal membrane fraction was then sedimented by centrifugation at 100,000 × *g* for 60 min. The pellet was resuspended in 0.25 M sucrose buffer (10 mM Tris-HCl, 1 mM EDTA, pH7.5) and homogenized using a Potter-Elvehjem homogenizer. Aliquots of membrane microsomes were rapidly frozen and stored at −80°C until analysis.

ATP Hydrolysis and Phosphate Release

The consumption of ATP was determined by the liberated inorganic orthophosphate, which forms a color complex with molybdate (10). We have modified an ATP hydrolysis assay based on phosphate-release determination using membrane microsome preparations to be carried out in a 96-well microplate(10). The microsomes were thawed on ice prior to diluting to 3.5 μg protein per well in ice-cold ATPase buffer (sodium ATP, 3 mM; KCl, 50 mM; MgSO4, 10 mM; dithiothreitol, 3 mM; Tris-HCl, 50 mM; pH 7.0) containing 0.5 mM EGTA (to inhibit Ca-ATPase), 0.5 mM ouabain (to inhibit the Na/K-ATPase), and 3 mM sodium azide (to inhibit the mitochondrial ATPase). The total incubation volume including the various inhibitors was 100 μl. The incubation reaction was initiated by transferring the plate from ice to 37°C and incubating for 30 min; the reaction was terminated by the addition of 50 μ12% SDS solution at room temperature, followed by the addition of 50 μl of a mixture solution of (equal volumes) of 18% fresh ascorbic acid in 1N HCl and 3% ammonium molybdate in 1 N HCl. After 4 min, 100 μl of a solution of 2% sodium citrate and 2% sodium meta-arsenite in 2% acetic acid was added to fix the color formation. After 30 min incubation at room temperature, the fixed released

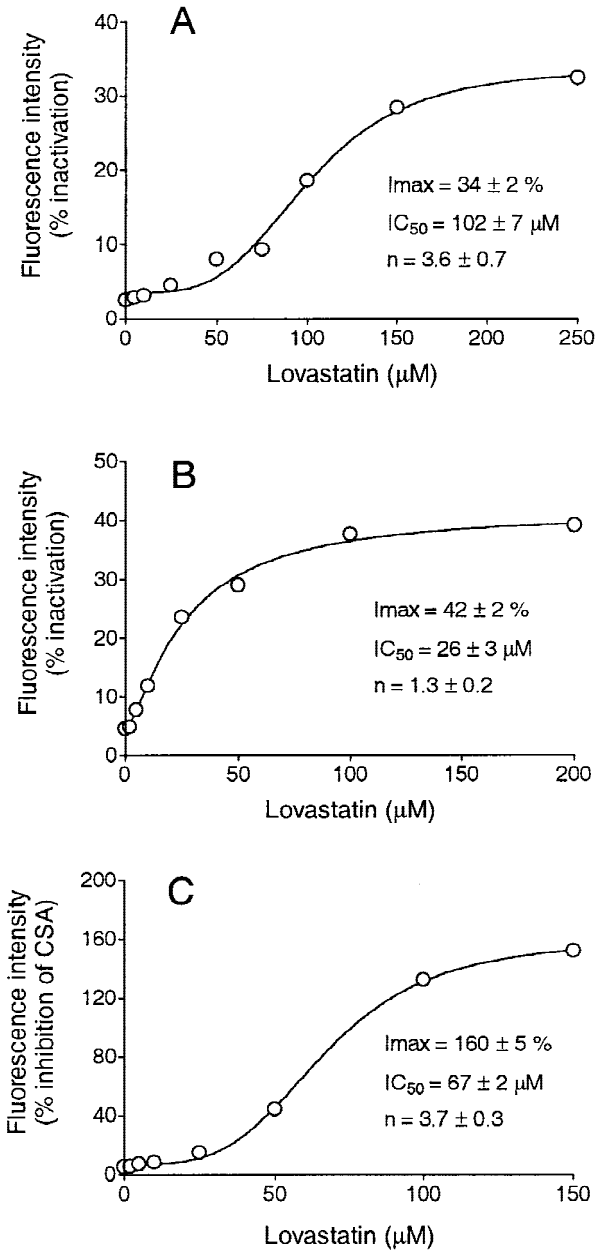

Fig. 1. Intracellular retention of daunorubicin (A and B) or rhodamine 123 (C) in CR1R12 cells (A) and G185 cells (B and C) vs. competing lovastatin concentration. Fluorescence intensity is expressed as relative fluorescence. The efflux phase or incubation was 30 min in all cases. The average number of cells per assay was 10,000. The function for the line through the data is the Hill equation: $v = V_{max}S^n/(K'+S^n)$. The parameters IC_{50} and the Hill coefficient along with the standard deviation are shown on the respective graphs.

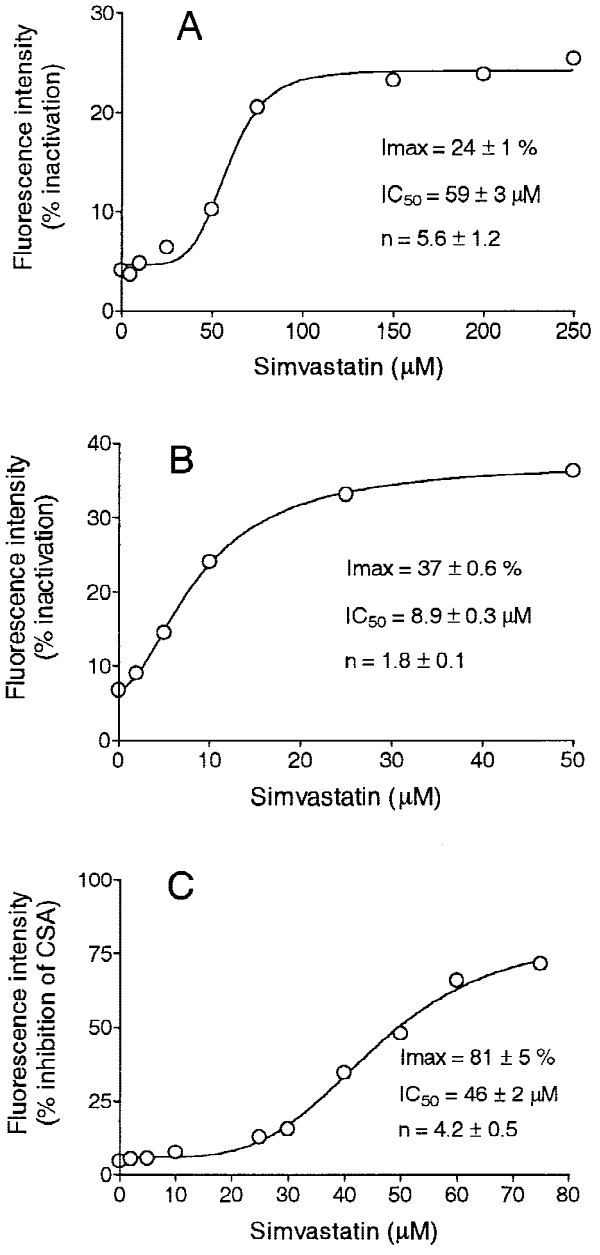

Fig. 2. Intracellular retention of daunorubicin (A and B) or rhodamine 123 (C) in CR1R12 cells (A) and G185 cells (B and C) vs. competing simvastatin concentration. Fluorescence intensity is expressed as relative fluorescence. The efflux phase or incubation was 30 min in all cases. The average number of cells per assay was 10,000. The function for the line through the data is the Hill equation: $v = V_{max}S^n/(K'+S^n)$. The parameters IC_{50} and the Hill coefficient along with the standard deviation are shown on the respective graphs.

phosphate was quantitated colorimetrically in a microplate reader (Bio-Tek FL600, VT) at 750 nm. The respective values for background with ATPase assay buffer alone were obtained in parallel and subtracted from the values for experimental samples. By comparison to a standard curve, the amount of phosphate released—and hence ATP consumed—was quantified. Water-insoluble drugs were dissolved in methanol; the maximum methanol concentration (2% v/v) was shown not to affect the ATPase activity.

RESULTS

As fluorescent substrates transported by P-gp, DNR and rhodamine 123 serve as markers for active transport function simply by measurement of fluorescence per cell (27). Herein we show that statins can effectively inhibit the P-gp-mediated transport of DNR or Rho. The IC_{50} (concentration at half maximum inhibition) can be determined from a simple function, as shown in Fig. 1, where the retained fluorescence is

measured for samples of viable cells by a flow cytometer at varying concentrations of statin. The concentration dependency of inhibition displayed a sigmoidal response curve (Fig. 1A), a consequence of cooperativity (8), with the Hill equation for allosteric interaction enzymes therefore being the appropriate function for fitting to the data: $v = V_{max}S^n/(K'+S^n)$. The IC_{50} of DNR transport in the CR1R12 cell line is ~102 μM, and lovastatin can achieve about 34% of transport inactivation. The efficacy of ~34% of complete inactivation is similar to many known P-gp substrates/inhibitors (27). For example, the commonly used positive control P-gp inhibitor verapamil inhibited DNR transport ~40% of the extent of complete inactivation by vanadate in the same experiment. As shown in Fig. 1B, the IC_{50} for lovastatin in the G185 cells (which express the gene product of human MDR1) is ~26 μM, which is four times as potent as the inhibition observed in the CR1R12 cell line overexpressing the rodent enzyme. Because these cell lines overexpress the respective transporter enzymes, the IC_{50} would be expected to be higher than under *in vivo* conditions, where far fewer copies of the enzyme would be contained per cell. The other fluorescent marker, Rho, is also retained in the presence of lovastatin with an IC_{50} ~ 67 μM (Fig. 1C). Simvastatin exhibits similar effects to lovastatin, as shown by Fig. 2A-C and summarized in Table I. The results with atorvastatin indicate a much lower affinity for the transporter with an IC_{50} of roughly 300 μM (Figs. 3A–C); these are summarized in Table I. Pravastatin, however, has no effect on the active efflux of DNR or Rho in either cell line and is therefore not a significant inhibitor of Pgp function (Fig. 4).

ATP Hydrolysis

As ATP is consumed at a purported rate of about one or two per transport event, the hydrolysis of ATP represents transport rate or activity assay of function (28–31,10). The presence of lovastatin causes a concentration-dependent increase in the rate of ATP hydrolysis relative to baseline rate, which indicates that it is a comparatively rapid substrate for P-gp (Fig. 5A). The K_m is ~20 μM, and the V_{max} is ~2.9-fold above baseline, as indicated in Table II. Similarly, simvastatin exhibits a K_m at ~18 μM and a V_{max} of ~2.7 times baseline (Fig. 5B and Table II). Atorvastatin again exhibits a significantly lower affinity for the Pgp transporter with a K_m of ~84 μM, and it seems to be a remarkably slow substrate (7), for V_{max} is significantly below baseline activity (Fig. 5C). Figure 5d indicates that pravastatin may be a substrate of medium efficiency for the Pgp transporter, as there is a slight decrease in turnover rate below the baseline rate. These data suggest a K_m of ~2 μM (with large confidence limits due to error) and a V_{max} of about 60% of baseline (Table II). The ATP hydrolysis activity assay results are consonant with the transport function inhibition assays (described above) with the possible exception of pravastatin.

DISCUSSION

HMG-CoA reductase inhibitors (statins) are widely prescribed for the treatment of hypercholesterolemic patients (32). Our results demonstrate the inhibition of P-gp transport function by the most popular statins in a concentration-dependent manner with an IC_{50} of <10 μM for simvastatin. These experiments use the G185 cell line expressing far above normal quantities of the human P-gp transporter enzyme. ATP hydrolysis kinetics show that simvastatin interacts with P-gp with a half maximal saturation concentration of about 18 μM. Since simvastatin, lovastatin, and atorvastatin are known to bind to CYP3A4, and both CYP3A4 and P-gp share a similar substrate definition (diverse lipophilic structures of ~300–1000 MW)—and hence share most substrates, it is not surprising that these statins would bind to the substrate binding site of P-gp. Indeed, the structures typify the "type II unit" of 3 electron donor groups with a spatial separation of 4.6 ± 0.6 Å suggested by Seelig (33) as one of two general patterns for substrate recognition by P-gp (constructed from a structure activity relationship study of known substrates of P-gp).

Some clinical observations seem to implicate statin interaction with P-gp transporter. Lovastatin concentration increased more than 20-fold when co-administered with itraconazole (13,14), and cyclosporine treatment significantly increased lovastatin accumulation (15). Because itraconazole and cyclosporine are both inhibitors of Pgp and CYP3A4, it is difficult to discern the dominant avenue of interaction. Simvastatin oral bioavailability is also significantly increased by treatment with itraconazole or cyclosporin (13,16–18), and P-gp substrates erythromycin and verapamil have been reported to increase serum concentrations of simvastatin con-

Table I. Inhibition Parameters of Pgp-Mediated Transport of DNR or Rho in Rodent MDR Cell Line or Cell Line Transfected with Human MDR1[a]

Statin	Cell line	Marker	IC_{50}, μM	I_{max}, %	n (Hill coeff.)
Lovastatin	CR1R12	DNR	102 ± 7	34 ± 2	3.6 ± 0.7
Lovastatin	3T3-G185	DNR	26 ± 3	42 ± 2	1.3 ± 0.2
Lovastatin	3T3-G185	Rho	67 ± 2	160 ± 5	3.7 ± 0.3
Simvastatin	CR1R12	DNR	59 ± 3	24 ± 1	5.6 ± 1.2
Simvastatin	3T3-G185	DNR	9 ± 0.3	37–0.6	1.8 ± 0.1
Simvastatin	3T3-G185	Rho	46 ± 2	81 ± 5	4.2 ± 0.5
Atorvastatin	CF1R12	DNR	356 ± 25	5 ± 0.3	11 ± 5
Atorvastatin	3T3-G185	DNR	271 ± 15	39 ± 3	4.8 ± 1.1
Atorvastatin	3T3-G185	Rho	307 ± 14	32 ± 2	7.6 ± 2.2
Pravastatin	CR1R12	DNR	ne	ne	ne
Pravastatin	3T3-G185	DNR	ne	ne	ne
Pravastatin	3T3-G185	Rho	ne	ne	ne

[a] Data are nonlinear regression solutions to Hill function followed by standard error. I_{max} is a percent of complete inactivation by vanadate. Verapamil inhibited DNR transport 26% of inactivation by vanadate. Pravastatin had no effect on function and this noted with the 'ne' entry.

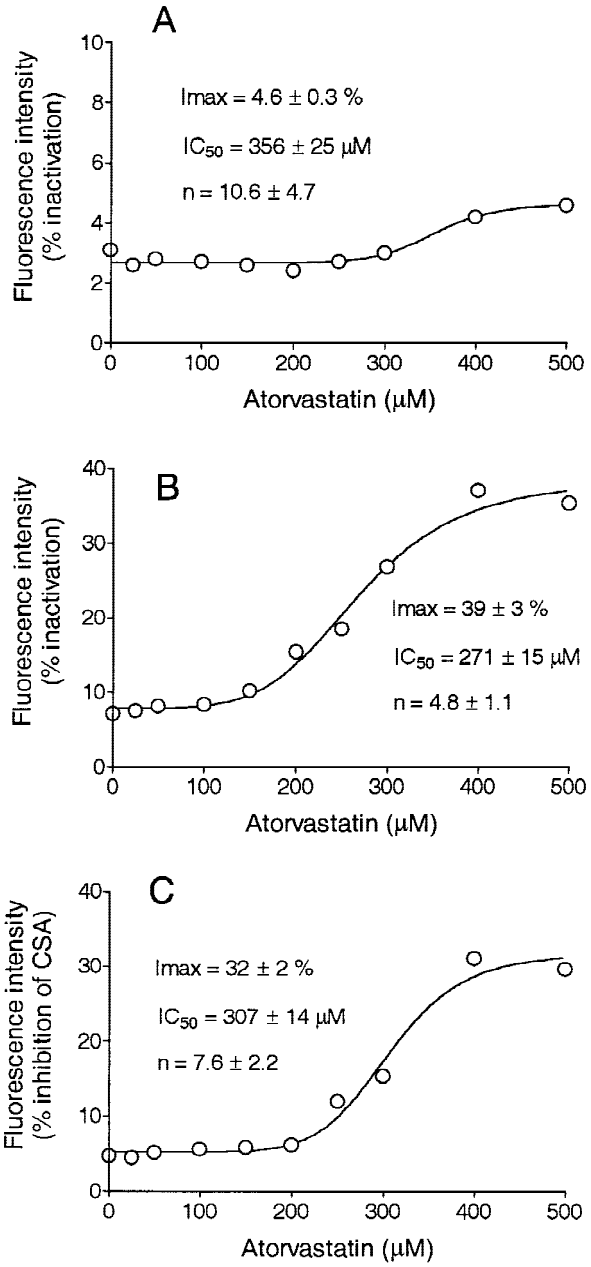

Fig. 3. Intracellular retention of daunorubicin (A and B) or rhodamine 123 (C) in CR1R12 cells (A) and G185 cells (B and C) vs. competing atorvastatin concentration. Fluorescence intensity is expressed as relative fluorescence. The efflux phase or incubation was 30 min in all cases. The average number of cells per assay was 10,000. The function for the line through the data is the Hill equation: $v = V_{max}S^n/(K'+S^n)$. The parameters IC_{50} and the Hill coefficient along with the standard deviation are shown on the respective graphs.

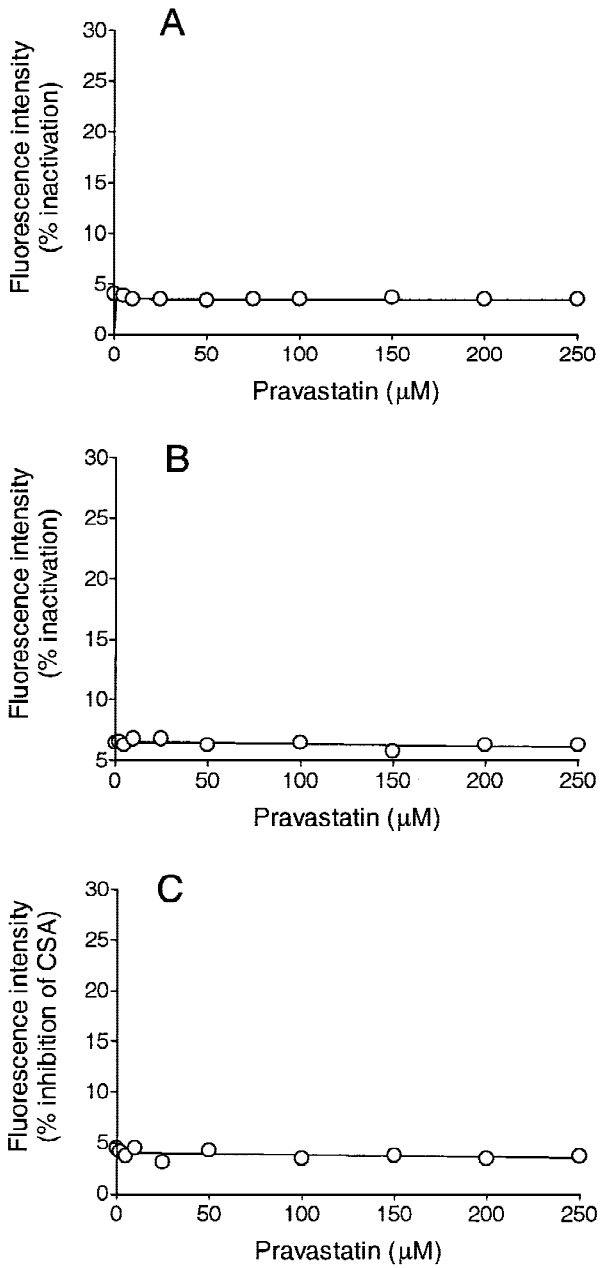

Fig. 4. Intracellular retention of daunorubicin (A and B) or rhodamine 123 (C) in CR1R12 cells (A) and G185 cells (B and C) vs. competing pravastatin concentration. Fluorescence intensity is expressed as relative fluorescence. The efflux phase or incubation was 30 min in all cases. The average number of cells per assay was 10,000. The function for the line through the data is the Hill equation: $v = V_{max}S^n/(K'+S^n)$. The parameters IC_{50} and the Hill coefficient along with the standard deviation are shown on the respective graphs.

siderably (34). Moreover, acute interactions have been observed with the administration of both digoxin, asserted to be a Pgp substrate/inhibitor, and simvastatin (19). Pravastatin exposure can increase in the presence of cyclosporine (15) despite a lack of significant interaction with CYP3A4 (16). However, itraconazole, a CYP3A4 and Pgp inhibitor, had no significant effect on the exposure to pravastatin (16), and pravastatin did not alter cyclosporine pharmacokinetics (15,35). Cyclosporine may interfere with pravastatin transport mediated by another ABC transporter (20, 25). Atorvastatin can clinically interact with ethinyl estradiol and erythromycin (21,36), both asserted to be inhibitors of CYP3A4 and Pgp. Additionally, coadministration of atorvastatin with digoxin will increase systemic exposure to digoxin (22).

Some in vitro studies suggest interactions of several statins with P-gp. Lovastatin-induced cytotoxicity in MDR1-expressing cell lines suggests that it is a substrate/inhibitor of the MDR1 (Pgp) transporter (23). Some evidence demon-

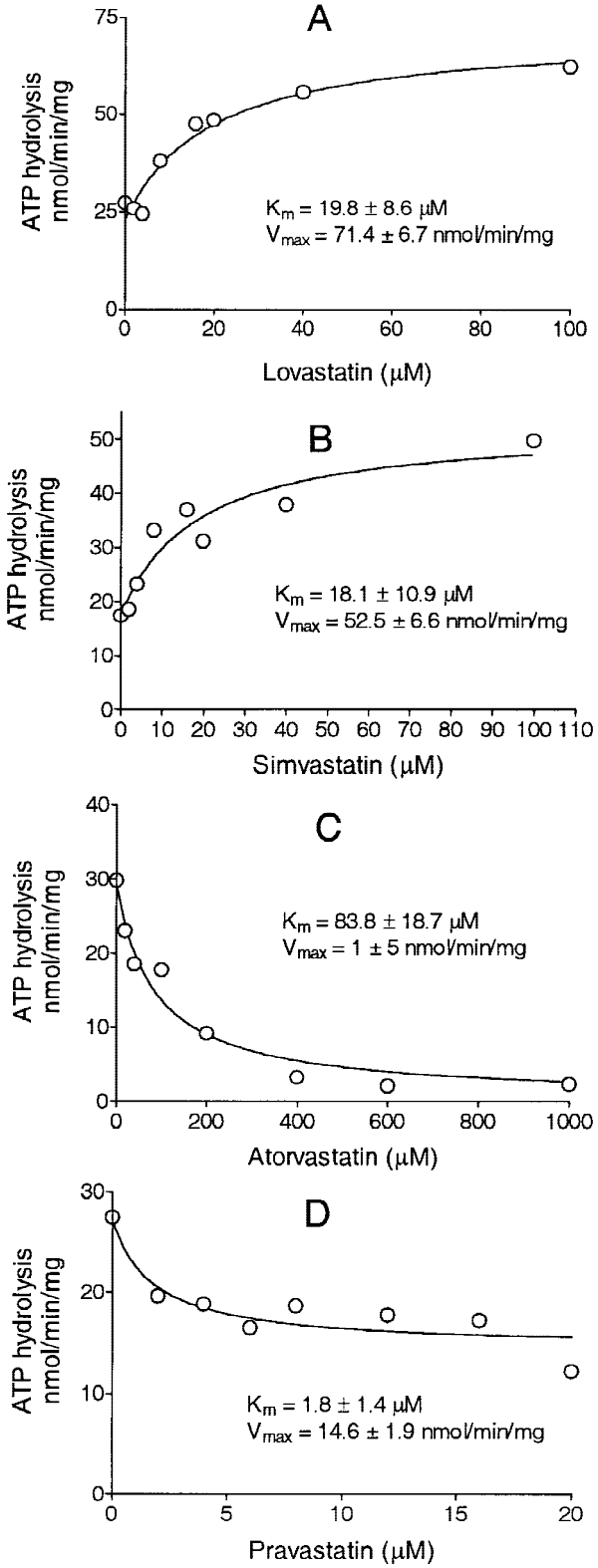

Fig. 5. P-gp-mediated ATP hydrolysis rates in the presence of lovastatin (A), simvasatin (B), atorvastatin (C), and pravastatin (D). The data is fit to a hyperbola and the V_{max} = 71 ± 7 nmol/min/mg membrane protein with a K_m = 20 ± 9 μM for lovastatin; the V_{max} = 53 ± 7 nmol/min/mg membrane protein with a K_m = 18 ± 11 μM for simvasatin; the V_{max} = 1 ± 5 nmol/min/mg membrane protein with a K_m = 84 ± 19 μM for atorvastatin; the V_{max} = 15 ± 2 nmol/min/mg membrane protein with a K_m = 2 ± 1 μM for pravastatin.

Table II. Kinetic Parameters of ATP Hydrolysis in the Presence of Statins

Statin	K_m, μM	V_{max}, nmol/min/mg
Lovastatin	20 ± 9	71 ± 7
Simvastatin	18 ± 11	53 ± 7
Atorvastatin	84 ± 19	1 ± 5
Pravastatin	2 ± 1	15 ± 2

strates that lovastatin may also be a substrate of MRP1 due to resistance of MRP1 presenting cells to the cytostatic effects of lovastatin (37). Asymmetric permeability to a given epithelium in Caco-2 cell monolayers of atorvastatin suggests that it may be a substrate of Pgp (22,24). Pravastatin, however, seems to be a substrate for MRP2 based on both *in vitro* experiments using canalicular membrane vesicles and *in vivo* using Eisai hyperbiliruminemic rats (25). Hence, administration of pravastatin may result in clinical interactions with compounds that are substrates for MRP2.

Unique among these statins, pravastatin does not inhibit the active transport of the marker substrates by Pgp; instead, it may be a low efficiency substrate that does not diffuse across the membrane and reenter the substrate binding site with sufficient effectiveness (38,39). Indeed, pravastatin has a carboxylate, where lovastatin and simvastatin have a lactone, as well as a hydroxyl, instead of a methyl, at the other end of the molecule. Therefore, because its diffusion rate across or through the cell membrane is expected to be less than lovastatin and simvastatin, pravastatin may not readily reassociate with the substrate binding site.

Several of these statins are further examples of effective inhibitors/substrates of both Pgp and CYP3A4. Because Pgp has a significant effect on absorption and disposition of orally administered drugs, its role might often be more significant than CYP3A4. Many, if not all, of the known clinical interactions with statins are with compounds that are substrates of Pgp, which therefore may be the significant cause of the clinical interactions. As several of these statins effectively inhibit the function of Pgp, clinical drug interactions should be expected with other Pgp substrates.

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