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Research Paper

Effects of Acid and Lactone Forms of Eight HMG-CoA Reductase Inhibitors on CYP-Mediated Metabolism and MDR1-Mediated Transport

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Purpose. With the growing clinical usage of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins), the number of reports concerning serious drug–drug interaction has been increasing. Because recent studies have shown that conversion between acid and lactone forms occurs in the body, drug–drug interaction should be considered on both acid and lactone forms. Thus, we investigated the inhibitory effects of acid and lactone forms of eight statins, including one recently withdrawn, cerivastatin, and two recently developed, pitavastatin and rosuvastatin, on cytochrome P450 (CYP) 2C8, CYP2C9, and CYP3A4/5 metabolic activities and multidrug resistance protein 1 (MDR1) transporting activity.

Methods. The inhibitory effects of statins on CYP metabolic activities and MDR1 transporting activity were investigated using human liver microsomes and MDR1-overexpressing LLC-GA5-COL150 cells, respectively.

Results. The acid forms had minimal inhibitory effects on all CYP activities tested, except for fluvastatin on CYP2C9-mediated tolbutamide 4-hydroxylation (IC_{50} = 1.7 μM) and simvastatin on CYP3A4/5-mediated paclitaxel 3-hydroxylation (12.0 μM). Lactone forms showed no or minimal inhibitory effects on CYP2C8, CYP2C9, and CYP2C19 activities, except for rosuvastatin on the CYP2C9 activity (20.5 μM), whereas they showed stronger inhibitory effects on the CYP3A4/5 activity with the rank order of atorvastatin (5.6 μM), cerivastatin (8.1 μM), fluvastatin (14.9 μM), simvastatin (15.2 μM), rosuvastatin (20.7 μM), and lovastatin (24.1 μM). Pitavastatin and pravastatin had little inhibitory effect, and a similar order was found also for testosterone 6β-hydroxylation. MDR1-mediated transport of [^{3}H]digoxin was inhibited only by lactone forms, and the rank order correlated with that of inhibitory effects on both CYP3A4/5 activities. Inhibitory effects on MDR1 activity, and on both CYP3A4/5 activities, could be explained by the lipophilicity; however, a significant correlation was found between the lipophilicity and inhibitory effects on CYP2C8-mediated paclitaxel 6α-hydroxylation.

Conclusions. We showed the difference between the acid and lactone forms in terms of drug interaction. The lipophilicity could be one of the important factors for inhibitory effects. In the case of statins, it is important to examine the effects of both forms to understand the events found in clinical settings, including the pleiotropic effects.

KEY WORDS: 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins); CYP; drug–drug interaction; MDR1.

INTRODUCTION

Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (statins) are widely used in the treatment of hypercholesterolemia and mixed dyslipidemias to reduce the risk of coronary heart disease and stroke through lowering levels of low-density lipoprotein and triglyceride. Additionally, recent reports have suggested that statins have an anti-inflammatory effect (1) and induce the production of nitric oxide (2), the so-called pleiotropic effects, resulting in a reduction in the risk of coronary heart disease. Based on the accumulation of evidence obtained *in vitro* and in clinical settings, statins are now being tried for other diseases, including Alzheimer's disease, cancer, and osteoporosis (3). As statins come to be used more frequently to treat complicated diseases, one should use them more carefully paying attention to drug–drug interactions, which raise the risk of adverse events (4). In 2001, cerivastatin was withdrawn from the market because of rhabdomyolysis found especially in patients coprescribed gemfibrozil. It has been proved that gemfibrozil elevated cerivastatin concentration with 5.6-fold for AUC of parent form and 4.4-fold for that of lactone form (5). The mechanisms have been revealed that

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gemfibrozil and its glucuronide inhibit hepatic uptake of cerivastatin mediated by organic anion transporting polypeptide 2 and hepatic metabolism mediated by CYP2C8 (6,7). In addition, monotherapy of cerivastatin has been reported to raise incidences of rhabdomyolysis (4), suggesting that cerivastatin itself would have higher potency to cause rhabdomyolysis.

Drug–drug interactions have been well investigated in terms of drug-metabolizing enzymes, especially cytochrome P450 (CYP) enzymes, among which CYP3A4 is considered the most important because it has a major role in the metabolism of many drugs (8). Recently, drug transporters have been recognized as another class of key molecules affecting a drug disposition (9–11). A number of drug transporters have been cloned and characterized, and the best characterized is multidrug resistance protein 1 (MDR1; P-glycoprotein). MDR1 was originally cloned in multidrug-resistant cancer cells (12,13), but it has been revealed that MDR1 is expressed in normal tissue (14,15) and involved in drug–drug interaction (16–20). It has been serendipitously noted that CYP3A4 and MDR1 show significant overlap in substrate or inhibitor specificity, and it has been also proposed that MDR1 would regulate the access of drugs to CYP3A4 in the intestine (21–24).

In this study, we compared the inhibitory effects of eight statins, including one recently withdrawn from the market, cerivastatin, and the recently developed, pitavastatin and rosuvastatin, on CYP2C8, CYP2C9, CYP2C19, and CYP3A4/5 activities and MDR1 activity using human liver microsomes and MDR1-overexpressing LLC-GA5-COL150 cells, respectively. Lovastatin and simvastatin are of a lactone form, whereas others are of an acid form, but both forms are found in human plasma after their administration (5,25–30), and the effects of both these forms of statins were examined independently herein to understand and estimate the possibility of drug interaction of statins.

MATERIALS AND METHODS

Materials

Acid forms of atorvastatin, cerivastatin, fluvastatin, pravastatin, rosuvastatin, and simvastatin and lactone forms of lovastatin and simvastatin were kindly provided by Sankyo Co. Ltd. (Tokyo, Japan), and all other statin acid and lactone forms were synthesized or extracted from products and purified by Kowa Co. Ltd. (Tokyo, Japan). Testosterone, tolbutamide, and colchicine were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Paclitaxel and digoxin were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). 4-Hydroxyl tolbutamide, *S*-mephenytoin, 4′-hydroxyl *S*-mephenytoin, 6α-hydroxyl paclitaxel, 3-hydroxyl paclitaxel, and 6β-hydroxyl- testosterone were purchased from Ultrafine Ltd. (Manchester, UK). [^{14}C]Testosterone (2.00 GBq/mmol), [^{14}C]tolbutamide (2.28 GBq/mmol), [^{14}C]-*S*-mephenytoin (2.05 GBq/mmol), and [methoxy-^{14}C]inulin (303 MBq/mmol) were purchased from Amersham Biosciences (Little Chalfont, UK). [^{14}C]Paclitaxel (1.96 GBq/mmol) and [^{3}H]digoxin (866 GBq/mmol) were provided by Sigma-Aldrich Co. and New England Nuclear (Boston, MA, USA), respectively. All other chemicals were obtained commercially or were of the highest grade requiring no further purification.

Inhibitory Effects of Statins on Activities of CYP Enzymes in Human Liver Microsomes

Paclitaxel 6α-hydroxylation, tolbutamide 4-hydroxylation, and *S*-mephenytoin 4′-hydroxylation were used as probe reactions for CYP2C8, CYP2C9, and CYP2C19, respectively, in pooled human liver microsomes (BD-GENEST™, Becton, Dickinson and Co., Franklin Lakes, NJ, USA). In addition, paclitaxel 3-hydroxylation and testosterone 6β-hydroxylation were used to assess CYP3A4 activity. The activities of CYP enzymes were evaluated by a method reported previously (31,32). Briefly, the incubation mixture (final volume, 250 μl) contained 0.5–1.0 mg of microsomal protein/ml, 1.3 mM β-NADP^{+}, 3.3 mM G-6-P, and 0.4 U/ml of G6P-DH in 0.1 M phosphate buffer (pH 7.4). The reaction was started by adding the microsomal protein solution following 5 min of prewarming. The reaction was stopped by adding acetonitrile at the designated time. The substrate concentrations of tolbutamide, paclitaxel, *S*-mephenytoin, and testosterone were 40, 4, 100, and 50 μM, respectively. The inhibitory effects of statins were assessed by the presence of inhibitors at concentrations of 1, 3, 10, 30, and 100 μM throughout experiments. The metabolites formed were determined by the method of thin-layer chromatography-radioluminography (TLC-RLG) as previously reported (33–36). The formation rates of metabolites were evaluated as the activities of CYP enzymes.

Culture of LLC-PK$_1$ and LLC-GA5-COL150 Cells

LLC-GA5-COL150 cells were established by transfection of MDR1 cDNA into porcine kidney epithelial LLC-PK$_1$ cells (16,17). Both lines were maintained in a culture medium consisting of Medium 199 (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum (Lot no. AKH12368 or AMJ17247, HyClone, Logan, UT, USA) without antibiotics. For LLC-GA5-COL150 cells, 150 ng/ml of colchicine was added for the stable expression of MDR1. LLC-PK$_1$ (1.0×10^6 cells; 1.82×10^4 cells/cm^2) and LLC-GA5-COL150 (1.5×10^6 cells; 2.73×10^4 cells/cm^2) cells were seeded on plastic culture dishes (100-mm diameter) in 10 ml of culture medium. They were grown in a humidified atmosphere of 5% CO_2/95% air at 37°C and subcultured every 4 and 7 days, respectively, with a 0.02% ethylenediaminetetraacetic acid–0.05% trypsin solution (Invitrogen, Carlsbad, CA, USA).

Transepithelial Transport of [^{3}H]Digoxin With or Without Statins Across LLC-PK$_1$ and LLC-GA5-COL150 Cell Monolayers

The transepithelial transport of [^{3}H]digoxin across LLC-PK$_1$ and LLC-GA5-COL150 cell monolayers was examined as described previously (16,20). Basal-to-apical transport and apical-to-basal transport were assayed independently. Both cells were seeded onto Transwell® (Cat. No. 3414, Corning

Costar, Cambridge, MA, USA) at a density of 2.0×10^6 cells/well (4.26×10^5 cells/cm^2) and 2.4×10^6 cells/well (5.11×10^5 cells/cm^2) for LLC-PK$_1$ and LLC-GA5-COL150 cells, respectively. They were cultured under a humidified atmosphere of 5% CO_2/95% air at 37°C for 3 days. At 3 h before the start of transport experiments, the culture medium was renewed. The transport experiment was initiated by replacement of the culture medium on the donor side with 2 ml of fresh culture medium containing [^{3}H]digoxin (100 nM, 18.5 kBq/ml) together with [methoxy-^{14}C]inulin (6.0 μM, 1.85 kBq/ml) and that on the receiver side with 2 ml of fresh culture medium. The monolayers were incubated at 37°C, and 25-μl aliquots were taken from the receiver side at 1, 2, and 3 h. The paracellular leakage estimated by the transport of inulin was less than 0.4% per hour in all experiments. The inhibitory effects of acid and lactone forms of statins were evaluated by adding inhibitors to both sides from 1 h before through 3 h after the experiment was initiated. The radioactivity of samples was determined by liquid scintillation counting (LSC-5100, Aloca Co. Ltd., Tokyo, Japan). In the assessment, the net basal-to-apical transport was calculated by subtracting the apical-to-basal transport from the basal-to-apical transport of [^{3}H]digoxin, and the ratio of net basal-to-apical transport with a statin to that without was calculated.

Calculation of the Logarithm of the Octanol/Water Partition Coefficient

To assess the impact of lipophilicity on inhibitory potency, we investigated the relationship between the calculated logarithm of the octanol/water partition coefficient (cLog *P*) of statins and IC_{50} values for CYP and MDR1 activities. cLog *P* values were calculated with Crippen's fragmentation method (37) using CS ChemDraw Ultra ver. 5.0 (Cambridge Soft Corp., Cambridge, MA), except the cLog *P* of rosuvastatin, which cannot be calculated using Crippen's method because of its structure.

Statistical Analysis

Spearman's correlation test was used for the correlation among the IC_{50} values for CYP activities and MDR1 activity and the values of cLog *P* of the acid and lactone forms of eight statins, giving a correlation coefficient ρ and associated probability *p*.

RESULTS

Table I lists the values for the IC_{50} of acid and lactone forms of eight statins for CYP2C8, CYP2C9, CYP2C19, and CYP3A4/5 metabolic activities investigated using human liver microsomes. Acid forms had minimal inhibitory effects on all tested CYP activities, except for fluvastatin on CYP2C9-mediated tolbutamide 4-hydroxylation (IC_{50} = 1.7 μM) and simvastatin on CYP3A4/5-mediated paclitaxel 3-hydroxylation (12.0 μM). Lactone forms showed no or minimal inhibitory effects on CYP2C8, CYP2C9, and CYP2C19 activities, except for rosuvastatin on CYP2C9-mediated tolbutamide 4-hydroxylation (20.5 μM), whereas they showed stronger inhibitory effects on CYP3A4/5-mediated paclitaxel 3-hydroxylation with a rank order of atorvastatin (5.6 μM), cerivastatin (8.1 μM), fluvastatin (14.9 μM), simvastatin (15.2 μM), rosuvastatin (20.7 μM), and lovastatin (24.1 μM).

Table I. IC_{50} Values for CYP-Mediated Metabolic Activities and MDR1-Mediated Transporting Activity for Eight Statins

		IC_{50} values (μM)					
Statins	Model substrates	Paclitaxel 6α-hydroxylation, CYP2C8	Tolbutamide 4-hydroxylation, CYP2C9	*S*-Mephenytoin 4-hydroxylation, CYP2C19	Testosterone 6β-hydroxylation, CYP3A4/5	Paclitaxel 3-hydroxylation, CYP3A4/5	Digoxin net B-to-A transport, MDR1
Atorvastatin	Acid form[a]	38.4	>100	>100	>100	74.6	>100
	Lactone form	28.8	61.0	>100	9.7	5.6	15.1
Cerivastatin	Acid form[a]	29.8[b]	>100	>100	>100	>100[b]	>100
	Lactone form	44.3[b]	42.9	>100	80.7	8.1[b]	28.2
Fluvastatin	Acid form[a]	70.2	1.7[b]	>100	>100	>100	>100
	Lactone form	55.4	81.8[b]	>100	48.1	14.9	>100
Lovastatin	Acid form	74.6	>100	>100	>100	>100	>100
	Lactone form[a]	79.9	>100	>100	26.9	24.1	44.5[c]
Pitavastatin	Acid form[a]	57.0	>100[b]	>100	>100	>100	>100
	Lactone form	50.5	>100[b]	>100	>100	67.2	34.9
Pravastatin	Acid form[a]	>100	>100	>100	>100	>100	>100[c]
	Lactone form	99.3	>100	>100	>100	73.7	>100
Rosuvastatin	Acid form[a]	>100[b]	>100[b]	>100	>100	>100[b]	>100
	Lactone form	32.5[b]	20.5[b]	>100	82.7	20.7[b]	>100
Simvastatin	Acid form	51.5	>100	>100	79.0	12.0	>100
	Lactone form[a]	44.1	>100	>100	76.8	15.2	59.6[c]

CYP, cytochrome P450.
[a] The form contained in oral prescriptions.
[b] Already reported in (32).
[c] Already reported in (20).

Table II. Spearman's Correlation Coefficient (ρ) and Associated Probability (p) for the Correlation Among the Values of cLog P and IC_{50} for CYP-Mediated Metabolic Activities and MDR1-Mediated Transporting Activity for Eight Statins

		Paclitaxel 6α-hydroxylation, CYP2C8	Tolbutamide 4-hydroxylation, CYP2C9	Testosterone 6β-hydroxylation, CYP3A4/5	Paclitaxel 3-hydroxylation, CYP3A4/5	Digoxin net B-to-A transport, MDR1
cLog P	ρ	−0.943	−0.358	−0.302	−0.497	−0.546
	p	<0.001	0.209	0.294	0.071	0.044
	n	14	14	14	14	14
Paclitaxel	ρ		0.336	0.347	0.496	0.354
6α-hydroxylation,	p		0.203	0.188	0.051	0.178
CYP2C8	n		16	16	16	16
Tolbutamide	ρ			0.331	0.382	0.183
4-hydroxylation,	p			0.210	0.144	0.498
CYP2C9	n			16	16	16
Testosterone	ρ				0.852	0.560
6β-hydroxylation,	p				<0.001	0.024
CYP3A4/5	n				16	16
Paclitaxel	ρ					0.621
3-hydroxylation,	p					0.010
CYP3A4/5	n					16

Pitavastatin and pravastatin hardly inhibited the activities with higher IC_{50} values. CYP3A4/5-mediated testosterone 6β-hydroxylation was inhibited by atorvastatin with an IC_{50} of 9.7 μM. The lactone forms of other statins had higher IC_{50} values, but the rank order was similar to that obtained for CYP3A4/5-mediated paclitaxel 3-hydroxylation.

Table I also lists the IC_{50} values for MDR1 transporting activity, as assessed using the MDR1-overexpressing cell line LLC-GA5-COL150 and a typical MDR1 substrate [^{3}H]digoxin. MDR1-mediated transport of [^{3}H]digoxin was inhibited only by lactone forms in the rank order of atorvastatin (15.1 μM), cerivastatin (28.2 μM), pitavastatin (34.9 μM), lovastatin (44.5 μM), and simvastatin (59.6 μM). Fluvastatin, pravastatin, and rosuvastatin showed no inhibition even at 100 μM.

Table II lists the Spearman's correlation coefficient (ρ) and associated probability (p) for the correlations among the values of the IC_{50} for and cLog P of acid and lactone forms. The cLog P values of seven statins (acid form/lactone form) are 5.05/5.58, 4.72/5.26, 3.79/4.32, 3.15/3.68, 4.09/4.63, 1.69/2.23, and 3.85/4.39 for atorvastatin, cerivastatin, fluvastatin, lovastatin, pitavastatin, pravastatin, and simvastatin, respectively. Those for rosuvastatin cannot be calculated with Crippen's method because of their structures. All of the lactone forms are more lipophilic than the corresponding acid forms. The rank order of the inhibitory effects on MDR1 activity was correlated with those of the inhibitory effects on both CYP3A4/5 activities obtained by paclitaxel 3-hydroxylation ($\rho = 0.621$, $p = 0.010$) and testosterone 6β-hydroxylation ($\rho = 0.560$, $p = 0.024$). Inhibitory effects on MDR1 activity were explained by the lipophilicity (Fig. 1; $\rho = -0.546$, $p = 0.044$). However, there was no correlation between those on both CYP3A4/5 activities and the lipophilicity ($\rho = -0.497$, $p = 0.071$ and $\rho = -0.302$, $p = 0.294$, respectively). It is noted

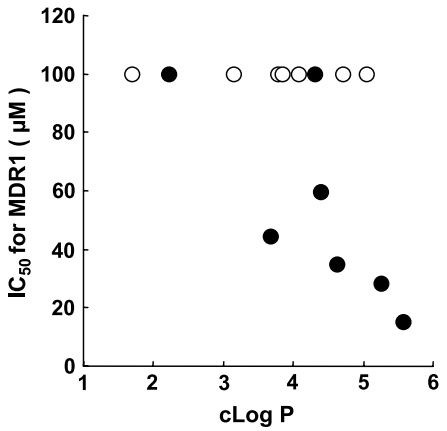

Fig. 1. Relationship between the values of cLog P and IC_{50} for MDR1 of eight statins. Closed and open circles represent lactone and acid forms, respectively. A significant correlation was found with $\rho = -0.546$ and $p = 0.044$.

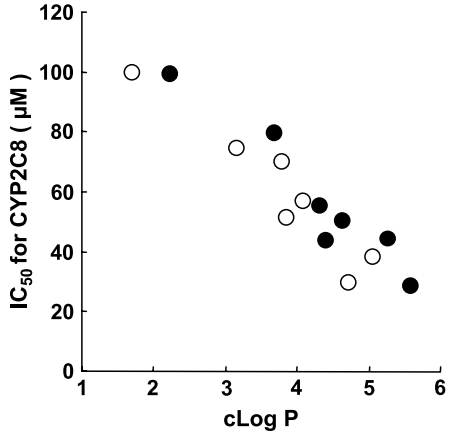

Fig. 2. Relationship between the values of cLog P and IC_{50} for CYP2C8 of eight statins. Closed and open circles represent lactone and acid forms, respectively. A significant correlation was found with $\rho = -0.943$ and $p < 0.001$.

Table III. Pharmacokinetic Parameters of Eight Statins

Statin		cLog *P*	C_{max} (ng/ml)	AUC (ng h/ml)	AUC ratio	$T_{1/2}$ (h)
Atorvastatin[a]	Acid form	5.05	12.7	61.4	1	7.8
	Lactone form	5.58	4.2	53.0	0.86	8.3
Cerivastatin[b]	Acid form	4.72	3.2	20.9	1	3.2
	Lactone form	5.26	0.27	1.9	0.09	4.8
Fluvastatin[c]	Acid form	3.79	197	324	1	2.4
	Lactone form	4.32	N. A.	N. A.	N. A.	N. A.
Lovastatin[d]	Acid form	3.15	17.6	76.9	1	2.5
	Lactone form	3.68	7	28.1	0.37	2.5
Pitavastatin[e]	Acid form	4.09	81.4	194.2	1	9.0
	Lactone form	4.63	49.5	269.3	1.39	6.1
Pravastatin[a]	Acid form	1.69	45.3	112.3	1	2
	Lactone form	2.23	1.6	3.3	0.03	N. A.
Rosuvastatin[f]	Acid form	N. C.	49.5	410	1	17.1
	Lactone form	N. C.	7.1	110	0.27	20.5
Simvastatin[g]	Acid form	3.85	3.1	21.7	1	2.8
	Lactone form	4.39	15.6	46.6	2.15	3.4

N. A.: Not available; N. C.: not calculated.
[a] PK parameters are from Lilja *et al.* (26).
[b] PK parameters are from Backman *et al.* (5).
[c] PK parameters are from Kivistö *et al.* (27).
[d] PK parameters are from Kantola *et al.* (28).
[e] PK parameters are from Ando *et al.* (29).
[f] PK parameters are from Schneck *et al.* (30).
[g] PK parameters are from Lilja *et al.* (25).

that a strong correlation was found between the lipophilicity and that on CYP2C8-mediated paclitaxel 6α-hydroxylation (Fig. 2; $\rho = -0.943$, $p < 0.001$).

DISCUSSION

With the growing clinical usage of statins, the number of reports concerning serious drug–drug interaction has been increasing. Fluvastatin and rosuvastatin enhanced the anticoagulant effect of warfarin (38,39). Atorvastatin has no effect on warfarin efficacy (40) but reduces the antiplatelet effect of clopidogrel (41). Atorvastatin and rosuvastatin elevate the blood concentration of cyclosporine and ethynyl-estradiol, respectively (42,43). Warfarin is mainly metabolized by CYP2C9, and CYP3A4 is understood to be responsible for the transformation of a prodrug of clopidogrel into an active metabolite and the metabolism of cyclosporine or ethynyl-estradiol. Atorvastatin, fluvastatin, and rosuvastatin are of the acid form, and atorvastatin and rosuvastatin have no or minimal effects on CYP2C9 or CYP3A4/5 activities (Table I). However, recently conducted clinical investigations have suggested a conversion from the acid to lactone forms and the opposite conversion after the administration (5,25–30). As summarized in Table III, the AUC ratio of the acid to lactone form varies from 0.03 for pravastatin to 2.15 for simvastatin, indicating that both forms of all statins except pravastatin should be considered in regard with drug–drug interaction. Herein, we elucidated that the lactone forms of atorvastatin and rosuvastatin inhibit CYP2C9 or CYP3A4/5 activities (Table I). Taken together, the recently reported drug interaction of atorvastatin or rosuvastatin can be explained by the actions of the lactone form, which can be transformed in the body, and not by that of the acid form, which is that in the medicine (26,30).

In this study, we clearly showed the difference between the acid and lactone forms of statins in terms of interaction with CYPs and MDR1. The results could be explained by the difference in chemical structure, but as shown in Table II and in Figs. 1 and 2, lipophilicity is a key factor in the affinity for them. Generally, it is well accepted that CYPs convert lipophilic substances to hydrophilic ones, and in turn, it is not surprising that the affinity for CYPs is defined by the lipophilicity. For MDR1, Tanaka *et al.* (44) have reported that the MDR1-mediated interaction between daunorubicin and cyclosporine analogs is defined by lipophilicity. To our knowledge, this is the first study to show the relationship between lipophilicity and the effects on CYP2C8 activity (Table I, Fig. 2). In the last 5 years, it has been recognized that CYP2C8 is an important CYP enzyme because the substrates of CYP2C8 are distinct from those of other members of CYP2C family, genetic polymorphisms affect the disposition of CYP2C8 substrate, and induction is mediated by the pregnane X receptor (45). The result is useful to avoid the development of new chemical entities, which is likely to cause drug–drug interaction-mediated inhibition of CYP2C8.

Recently, it has been elucidated that UDP-glucuronosyltransferase (UGT) 1A1, UGT1A3, and UGT2B7 are responsible for the conversion of statins from acid to lactone forms (36,46,47). Because genetic polymorphisms are reported, at least, for UGT1A1 and UGT2B7 (48), there will be ethnic differences in the interaction between statins and other drugs, and the magnitude of the interaction will depend on their genotypes. Here, we showed the difference between the acid and lactone forms in terms of drug interaction. In the case of statins, it is important to examine the effects of both

forms to understand the events found in clinical settings, including the pleiotropic effects.

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