Previous human studies agree that among numerous SNPs in the *SLCO1B1* gene, 521T→C (174Val→Ala) plays an important role in the transport capability, reducing hepatic uptake of pravastatin (Table 5) [103,104]. Because the target tissue of pravastatin is hepatocytes [186,187], subjects with this allele may exhibit reduced cholesterol-lowering effect of pravastatin due to lower pravastatin concentration in the hepatocytes, despite high plasma levels and AUC of pravastatin. At least two studies have been conducted to clarify this hypothesis. Tachibana-Iimori *et al.* [188] conducted a retrospective study on 66 patients who underwent treatment for hyperlipidaemia with HMG-CoA reductase inhibitors. They found that patients with the 521C allele showed an attenuated total-cholesterol-lowering effect compared with those homozygous for the 521T allele. Niemi *et al.* [189] investigated the association between polymorphism in the *SLCO1B1* and plasma concentrations of lathosterol and cholesterol up to 12 h after the intake of a single dose of pravastatin 40 mg in 41 healthy Caucasian subjects, and found that the plasma lathosterol level and lathosterol to cholesterol level ratio, markers of the rate of cholesterol synthesis *in vivo*, were significantly lower among the three heterozygous carriers of the *SLCO1B1*17* haplotype as compared with noncarriers. Both studies suggest that the 521T→C polymorphism modulates the lipid-lowering efficacy of HMG-CoA reductase inhibitors.

5. Conclusion

The polymorphism of genes encoding drug transporters is a useful marker to interpret large interindividual differences in the pharmacokinetics and response (pharmacodynamics) of clinically important drugs, and a great deal of effort is now being directed at assessing genotype–phenotype relationships not only in the clinical setting, but also at all stages of drug development. Numerous drug transporters, except the transporters described here, may also play an important role in the human body. Gene-knockout animals and expression cell systems are now available for the characterisation of basic traits such as substrate specificity, localisation and vectorial movement. Thus, in order to elucidate their *in vivo* functions more precisely, it seems appropriate to integrate the results from *in vitro* experiments/animal studies into the human study. Further refining of this integration will provide more precise and useful observations, allowing for truly genome-based scientific pharmacotherapy.

6. Expert opinion

Genetic polymorphisms have been identified in most known drug transporters. Some of these variants were shown to have an impact on pharmacokinetic and pharmacodynamic consequences in pharmacotherapy, but unfortunately, functional confirmation remains to be elucidated for most of these variants. We are now beginning to elucidate and understand the consequences of these variants in the human body. So far, except for a few cases (e.g., the *SLCO1B1* genotype and statins pharmacokinetics/pharmacodynamics), there are still discrepancies in the results of functional confirmation (i.e., phenotype and genotype relationship), thus necessitating some concerns for further investigations.

Controversial and confused observations relating to the *in vivo* pharmacokinetic relevance of the polymorphisms of some drug transporter genes (e.g., *ABCB1* and *ABCG2*) may have arisen from the nonspecific substrate drugs used in the various studies. For example, in the *ABCB1* polymorphism, although digoxin and fexofenadine have been used as probed drugs for P-gp function, these are also known to be substrates, at least for polymorphic *SLCO1B3* and *SLCO1B1*, respectively.

Despite considerable effort, it is difficult to find specific substrates to corresponding specific transporters because the substrate specificity of most transporters is extremely broad and shows substantial overlap between different members of the superfamily. For this perspective, multiple gene analysis of the network of genes involved in drug metabolism, transport, and response (e.g., receptors), is preferable. For example, previous *in vitro* experiments reported that at least two transporters, but no cytochrome P450s, are involved in the pharmacokinetics of pitavastatin; OATP1B1 for uptake into hepatocytes and BCRP for efflux into the bile and gut lumen [190]. A pharmacogenomic human study of pitavastatin conducted with polymorphisms in *SLCO1B1* and *ABCG2* is of interest. Again, in order to establish a pharmacokinetic gene network, the integration of *in vitro* and animal experiments into the human study is essential.

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Pharmacogenetic determinants of variability in lipid-lowering response to pravastatin therapy

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Abstract Pravastatin is mainly taken up from the circulation into the liver via organic anion-transporting polypeptide 1B1 (*SLCO1B1* gene product). We examined the contribution of genetic variants in the *SLCO1B1* gene and other candidate genes to the variability of pravastatin efficacy in 33 hypercholesterolemic patients. In the initial phase of pravastatin treatment (8 weeks), heterozygous carriers of the *SLCO1B1**15 allele had poor low-density lipoprotein cholesterol (LDL-C) reduction relative to non-carriers (percent reduction: –14.1 vs –28.9%); however, the genotype-dependent difference in the cholesterol-lowering effect disappeared after 1 year of treatment. Cholesterol 7α-hydroxylase (*CYP7A1*) and apolipoprotein E (*APOE*) are known to contribute to lipid metabolism. Homozygous carriers of the *CYP7A1* -204C allele or heterozygotes for both *CYP7A1* -204C and *APOE* ε4 alleles showed significantly poorer LDL-C reduction compared to that in other genotypic groups after 1 year of treatment (–24.3 vs –33.1%). These results suggest that the *SLCO1B1**15 allele is associated with a slow response to pravastatin therapy, and the combined genotyping of *CYP7A1* and *APOE* genes is a useful index of the lipid-lowering effect of pravastatin.

Keywords *SLCO1B1* · *CYP7A1* · *APOE* · Pravastatin · Cholesterol

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Introduction

Coronary heart disease is the leading cause of death worldwide. Several risk factors for cardiovascular disease are well known, especially increased low-density lipoprotein cholesterol (LDL-C) and decreased high-density lipoprotein cholesterol (HDL-C). Statins are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a rate-limiting enzyme in cholesterol biosynthesis. Lipid-lowering therapy by statins has the potential to improve outcomes in patients at risk for cardiovascular disease. Despite these large effects, interindividual variability in the response to statins has been observed in clinical situations (Pazzucconi et al. 1995). Previous studies have demonstrated that the mechanisms responsible for variability in the statin response are due, at least in part, to genetic factors. Most studies have focused on the association between variants (ε2, ε3 and ε4) in apolipoprotein E (*APOE*) gene, which is a primary ligand for the LDL receptor found on the liver, and the response to statins (Ojala et al. 1991; Ordovas et al. 1995). In addition, recent studies have demonstrated

that variants in cholesterol 7alpha-hydroxylase (*CYP7A1*) (Pullinger et al. 2002), *ABCG8* (Kajinami et al. 2004) and HMG-CoA reductase (*HMGCR*) (Chasman et al. 2004) are important determinants of the lipid response to statin therapy.

Pravastatin, a hydrophilic HMG-CoA reductase inhibitor, is taken up efficiently from the circulation into the liver by an active transport carrier system, but is not metabolized by CYP enzymes. Human organic anion-transporting polypeptide 1B1 (OATP1B1), transporter of pravastatin, is expressed on the basolateral membrane in the hepatocytes responsible for the hepatocellular uptake of pravastatin (Hsiang et al. 1999). The major site of cholesterol synthesis, the liver, is the main target organ of statins. Recently, Niemi et al. (2005) have shown that the *SLCO1B1**17 allele (containing -11187G>A, 388A>G and 521T>C) is associated with the decreased acute effect of pravastatin on cholesterol synthesis; however, the impact of *SLCO1B1* genotypes on the lipid-lowering response to pravastatin during long-term treatment has not been well investigated.

The aim of this study was to describe the influence of *SLCO1B1* genotypes on the lipid-lowering response to pravastatin in Japanese hypercholesterolemic patients. Furthermore, we evaluated the contribution of genetic variants in other candidate genes (*APOE*, *CYP7A1*, *ABCG8* and *HMGCR*) to the variability in pravastatin efficacy.

Materials and methods

Study design

We studied 33 patients (14 males and 19 females; mean age 62.3 years; age range 34–83 years) with hypercholesterolemia treated in Tottori University Hospital. All subjects were initially prescribed pravastatin (mean dose range 9.4 mg/day) between January 1997 and October 2004. We used the electronic medical database available in the hospital to obtain precise information on patients' backgrounds, laboratory tests, prescribed drugs and adverse events. We collected these data retrospectively for each patient for at least 1 year from the day pravastatin was administered. Patients with serious or uncontrolled renal or liver disease, no drug compliance, other hypolipidemic treatment or uncontrolled diabetes were excluded. The average body mass index (BMI), total cholesterol (TC) and LDL-C values in this study patients were 23.9 kg/m^2 (range 17.3–30.9 kg/m^2), 259.6 mg/dl (range 225.8–315.0 mg/dl) and 167.4 mg/dl (range 112.0–240.7 mg/dl), respectively. This study was approved by the Tottori University Ethics Committee, and informed consent was obtained from all individuals.

Genotyping

All subjects were genotyped for variants in the candidate genes involved in the pharmacokinetics and pharmacodynamics of pravastatin. Details of the genotyping and haplotyping of *SLCO1B1**1b (388A>G), *5 (521T>C) and *15 (388A>G and 521T>C) were described previously (Nishizato et al. 2003). The promoter variant (-11187G>A) in the *SLCO1B1* gene was determined with PCR–SSCP analysis. The *SLCO1B1* -11187G>A variant was observed as heterozygosity (0.212) in this patient group suggesting it was tightly linked to the *SLCO1B1**15 allele. The genotypes in *CYP7A1* (-204A>C) (Hubacek et al. 2003), *APOE* (ϵ2, ϵ3 and ϵ4) (Hixon and Vernier 1990) and *ABCG8* (55G>C) (Kajinami et al. 2004) were examined by previously described methods using PCR restriction fragment length polymorphism analysis. Genetic variants (SNP12 and 29) in the *HMGCR* gene were found as functional variants for variable response to statin therapy in the previous study (Chasman et al. 2004) as determined with PCR–SSCP analysis.

Statistical analysis

Comparisons between two groups were performed using a Student *t*-test and between more than two groups using ANOVA (with Tukey–Kramer multiple comparison test). A 5% level of probability was considered to be significant.

Results and discussion

The mean percent reductions from the baseline in TC and LDL-C values at 8 weeks post-treatment with pravastain were significantly smaller in heterozygous carriers of the *SLCO1B1**15 allele than in homozygous carriers of the *1a and *1b alleles (Fig. 1a, $P<0.05$). Also, the mean percent reduction from the baseline in TC values at 8 weeks post-treatment was significantly smaller in *SLCO1B1**15 carriers than in non-carriers (–9.8 vs –20.9%; $P<0.05$; Fig. 1b). A similar trend was observed in the LDL-C level (–14.1 vs –28.9%, $P<0.05$; Fig. 1b) even though the pravastatin daily dose (mean±SD; non-carriers: 9.4±2.9 mg, carriers:

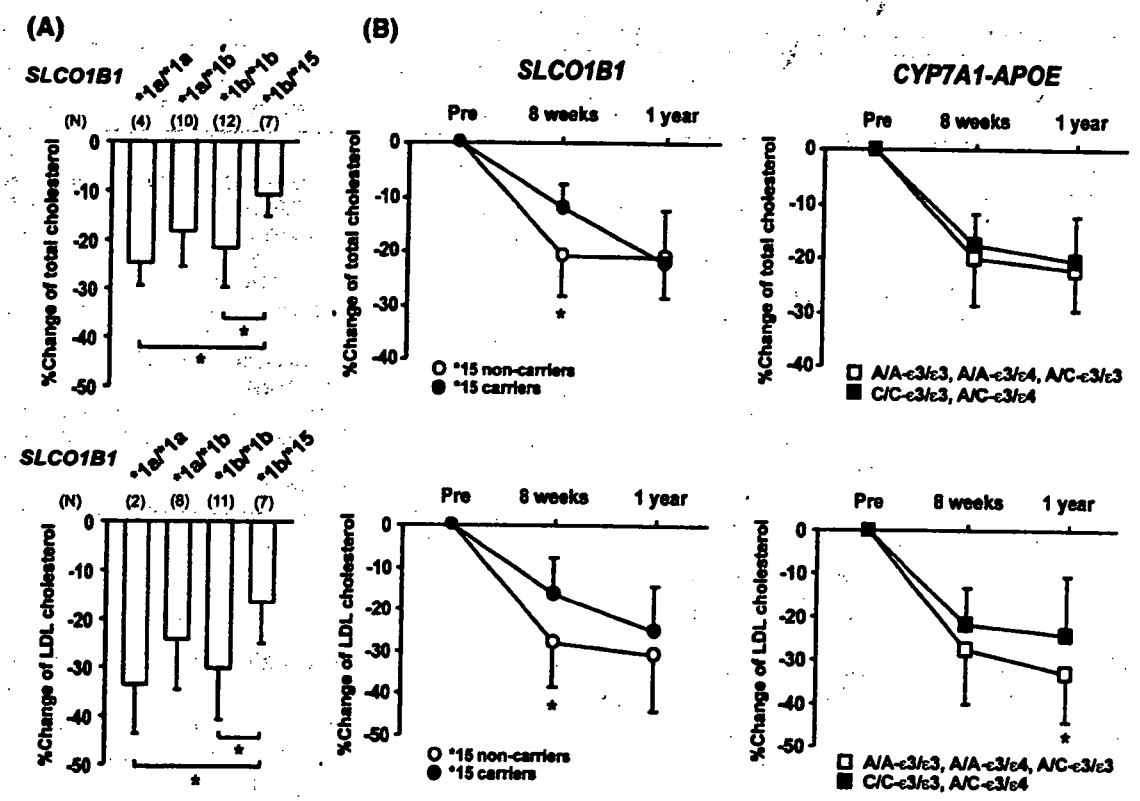

Fig. 1 a Influence of the *SLCO1B1* genotypes on percent reduction from baseline in TC and LDL-C values at 8 weeks after pravastatin treatment. **P*<0.05 when compared between the two groups using Tukey–Kramer multiple comparison test. b Influence of the *SLCO1B1*, *CYP7A1* and *APOE* genotypes on time course of percent reduction from baseline in TC and LDL-C value after pravastatin treatment. **P*<0.05 when compared between the two genotypes was analyzed with Student's *t*-test. Each value is the mean±SD

9.3±2.0 mg,) and BMI (non-carriers: 24.1±3.5 kg/m^2, carriers: 23.5±2.7 kg/m^2) were not significantly different between the two groups. In contrast, at 1 year post-treatment, there were no significant differences in the reduction of TC and LDL-C values between the two groups (Fig. 1b; Table 1).

In an in vitro experiment, Iwai et al. (2004) demonstrated that the transport activity of *SLCO1B1**15 allele is significantly decreased compared with that of the *SLCO1B1**1a or *1b allele using cDNA-transfected HEK293 cells. Previously, we found *SLCO1B1**15 allele was associated with higher plasma concentration of pravastatin, and the non-renal clearance of pravastatin in subjects with *SLCO1B1**1b/*15 and *15/*15 was reduced to 55 and 14% of *1b/*1b subjects, respectively (Nishizato et al. 2003). Thus, it is suggested that the *SLCO1B1**15 allele leads to an increase in plasma pravastatin concentrations but a reduction in the hepatocellular uptake of pravastatin, resulting in a decreased effect of pravastatin. However, interestingly, the genotype-dependent difference in this lowering effect disappeared after long-term treatment. Although its mechanism remains to be elucidated, one possible reason is that all of our patients with the *SLCO1B1**15 allele were heterozygotes for functionally active *1a or *1b alleles (Iwai et al. 2004). Thus, the lipid-lowering profiles in homozygotes for the *15 allele are of interest.

Multidrug resistance-associated protein 2 (MRP2/ABCC2) on the bile canalicular membrane is mainly involved in the biliary excretion of pravastatin (Matsushima et al. 2005). With regard to liver concentration of pravastatin, genetic polymorphisms of *MRP2* might affect response to pravastatin. However, *MRP2* variants have been observed at low frequency in Japanese (Itoda et al. 2002), and functional significance of these variants is not established. Therefore, association of *MRP2* genotypes should be analyzed by further studies.

We also examined the influence of the *CYP7A1* promoter (-204A/C) and *APOE* (ε2, ε3 and ε4) variants on the clinical outcome of pravastatin therapy. As shown in Fig. 1b and Table 1, the reduction from the baseline in LDL-C value at 1 year post-treatment was

Table 1 Association of *SLCO1B1*, *CYP7A1* and *APOE* genotypes with lipid changes

Gene	Genotype	Lipid concentrations (mg/dl)					
		N	Baseline	*N*	8 weeks	*N*	1 year
Total cholesterol							
*SLCO1B1*15*	Non-carriers	26	260.9±24.4	26	205.8±22.2	20	201.9±18.5
	Carriers	7	254.8±10.6	7	227.9±19.6	6	204.0±16.5
	P value		NS		<0.05		NS
CYP7A1-APOE	A/A-ε3/ε3, A/A-ε3/ε4, A/C-ε3/ε3	19	261.9±23.9	19	210.3±27.9	14	198.9±12.7
	C/C-ε3/ε3, A/C-ε3/ε4	14	256.4±20.1	14	210.7±16.0	12	206.0±22.3
	P value		NS		NS		NS
LDL cholesterol							
*SLCO1B1*15*	Non-carriers	22	170.7±27.4	22	124.0±20.7	17	115.1±23.9
	Carriers	7	157.0±29.3	7	132.0±32.7	6	110.5±10.9
	P value		NS		NS		NS
CYP7A1-APOE	A/A-ε3/ε3, A/A-ε3/ε4, A/C-ε3/ε3	19	168.6±34.4	19	124.0±29.9	12	106.3±20.6
	C/C-ε3/ε3, A/C-ε3/ε4	12	165.7±16.3	12	128.7±12.5	10	123.8±12.5
	P value		NS		NS		<0.05

Values are mean±SD

Statistical significance between the two genotypes was analyzed with Student's *t*-test

NS No significant difference

significantly decreased in carriers of A/A-ε3/ε3, A/A-ε3/ε4 or A/C-ε3/ε3 in *CYP7A1* and *APOE* genes compared with C/C-ε3/ε3 or A/C-ε3/ε4 carriers. There was no significant effect of genotypes (A/A-ε3/ε3, A/A-ε3/ε4 or A/C-ε3/ε3 vs C/C-ε3/ε3 or A/C-ε3/ε4) in the *CYP7A1* and *APOE* genes on pravastatin dose (10.0±2.9 vs 8.8±2.9 mg) and BMI (23.8±3.6 vs 24.5±3.0 kg/m^2). Only one patient was a heterozygous carrier of SNP12 in the *HMGCR* gene. However, no remarkable difference in the lipid-lowering effects was observed in this patient. Also, SNP29 in *HMGCR* and 55G>C in *ABCG8* were not detected.

In contrast to *SLCO1B1* gene, part of the interpatient variability in the efficacy of pravastatin after long-term treatment may be attributable to genetic variation, and combined genotyping of *CYP7A1* and *APOE* genes is useful for describing the lowering effects. Since the basal cholesterol synthesis rate is a key determinant for statin response, loss of CYP7A1 activity, which is involved in bile acid synthesis from cholesterol in the liver, may result in a poor response to statin treatment (Pullinger et al. 2002). A previous study has shown that the nucleotide sequence around position -204 negatively regulates CYP7A1 promoter activity (Cooper et al. 1997). Among the known variants, the *CYP7A1* -204A>C variant is expected to decrease promoter activity (Kajinami et al. 2005). Apolipoprotein E is known as one of the major determinants in lipoprotein metabolism. Previous studies (Ojala et al. 1991; Ordovas et al. 1995) demonstrated that the ε4 allele in primary hypercholesterolemia is associated with lower response to statin, when compared to ε2 and ε3 alleles, because the binding activity of ε4 allele to receptor is relatively higher than that of other alleles. These results suggest that decreased cholesterol 7alpha-hydroxylase activity and increased binding affinity of apolipoprotein E to LDL receptor enhance the intracellular cholesterol content in hepatocytes, resulting in lower HMG-CoA reductase activity, which may also lead to tolerance to statin treatment (Kajinami et al. 2005).

In conclusion, our results suggest that the *SLCO1B1*15* allele is associated with a slow response to pravastatin. Instead of *SLCO1B1*15*, combined genotyping of *CYP7A1* -204A>C and *APOE* ε4 variants may be useful for describing the long-term clinical outcomes of pravastatin. Further study is necessary to confirm the influence of genetic variants in these candidate genes on the lipid-lowering efficacy of pravastatin as well as other statins in a large sample size.

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Effects of organic anion transporting polypeptide 1B1 haplotype on pharmacokinetics of pravastatin, valsartan, and temocapril

Objective: Recent reports have shown that genetic polymorphisms in organic anion transporting polypeptide (OATP) 1B1 have an effect on the pharmacokinetics of drugs. However, the impact of *OATP1B1*1b* alleles, the frequency of which is high in all ethnicities, on the pharmacokinetics of substrate drugs is not known after complete separation of subjects with *OATP1B1*1a* and **1b*. Furthermore, the correlation between the clearances of OATP1B1 substrate drugs in individuals has not been characterized. We investigated the effect of genetic polymorphism of OATP1B1, particularly the **1b* allele, on the pharmacokinetics of 3 anionic drugs, pravastatin, valsartan, and temocapril, in Japanese subjects.

Methods: Twenty-three healthy Japanese volunteers were enrolled in a 3-period crossover study. In each period, after a single oral administration of pravastatin, valsartan, or temocapril, plasma and urine were collected for up to 24 hours.

Results: The area under the plasma concentration–time curve (AUC) of pravastatin in **1b/*1b* carriers (47.4 ± 19.9 ng · h/mL) was 65% of that in **1a/*1a* carriers (73.2 ± 23.5 ng · h/mL) ($P = .049$). Carriers of **1b/*15* (38.2 ± 15.9 ng · h/mL) exhibited a 45% lower AUC than **1a/*15* carriers (69.2 ± 23.4 ng · h/mL) ($P = .024$). In the case of valsartan we observed a similar trend as with pravastatin, although the difference was not statistically significant (9.01 ± 3.33 μg · h/mL for **1b/*1b* carriers versus 12.3 ± 4.6 μg · h/mL for **1a/*1a* carriers [$P = .171$] and 6.31 ± 3.64 μg · h/mL for **1b/*15* carriers versus 9.40 ± 4.34 μg · h/mL for **1a/*15* carriers [$P = .213$]). The AUC of temocapril also showed a similar trend (12.4 ± 4.1 ng · h/mL for **1b/*1b* carriers versus 18.5 ± 7.7 ng · h/mL for **1a/*1a* carriers [$P = .061$] and 16.4 ± 5.0 ng · h/mL for **1b/*15* carriers versus 19.0 ± 4.1 ng · h/mL for **1a/*15* carriers [$P = .425$]), whereas that of temocaprilat (active form of temocapril) was not significantly affected by the haplotype of OATP1B1. Interestingly, the AUC of valsartan and temocapril in each subject was significantly correlated with that of pravastatin ($R = 0.630$ and 0.602, $P < .01$). The renal clearance remained unchanged for each haplotype for all drugs.

Conclusion: The major clearance mechanism of pravastatin, valsartan, and temocapril appears to be similar, and *OATP1B1*1b* is one of the determinant factors governing the interindividual variability in the pharmacokinetics of pravastatin and, possibly, valsartan and temocapril. (Clin Pharmacol Ther 2006;79:427-39.)

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The administration of the same dose of a drug sometimes results in large interindividual differences in pharmacokinetics and subsequent pharmacologic and toxicologic effects. The pharmacokinetics of certain drugs are dominated by absorption, disposition, metabolism, and elimination, and many molecules, such as metabolic enzymes and transporters, have been reported to be involved in each process. Recently, polymorphisms in each molecule have been identified, and many in vitro and clinical studies have demonstrated that some of them are associated with a change in the expression and function of molecules and the pharmacokinetics of drugs. Although there is much information regarding metabolic enzymes such as cytochrome P450 (CYP) and phase II conjugation enzymes, the clinical significance of the genetic polymorphisms in transporters is not well understood.

Organic anion transporting polypeptide (OATP) 1B1 (formerly known as OATP-C or OATP2) is exclusively expressed in the liver and located on the basolateral membrane.[1–3] Some reports have indicated that OATP1B1 can transport a wide variety of compounds including clinically important drugs such as 3-hydroxy-3-methylglutaryl–coenzyme A reductase inhibitors,[1,4,5] which suggests that OATP1B1 may be responsible for the hepatic uptake of various kinds of anionic drugs, which efficiently accumulate in liver. Hepatic clearance consists of intrinsic clearances of hepatic uptake, sinusoidal efflux, metabolism, and biliary excretion. From the viewpoint of pharmacokinetics, a change in the uptake process will directly affect the overall hepatic clearance, regardless of the absolute values of each intrinsic clearance.[6] Therefore genetic polymorphisms in OATP1B1 may have an effect on the hepatic clearance of OATP1B1 substrates.

Several genetic polymorphisms in OATP1B1 have been reported, and in vitro studies have shown that some of them reduce the transport capability of several substrates in OATP1B1 variant–expressing cells.[7–9] Among these, previous studies have focused on 2 mutations, Asn130Asp and Val174Ala, because they are frequently observed in all ethnic groups investigated previously and their allele frequencies show some ethnic differences,[9,10] which may cause an ethnic difference in the pharmacokinetics of OATP1B1 substrates. Interestingly, Nishizato et al[10] demonstrated that Val174Ala was tightly linked with Asn130Asp and formed a haplotype referred to as *OATP1B1*15* in Japanese subjects. In addition, after oral administration of pravastatin, healthy Japanese volunteers with the **15* allele showed an increase in the area under the plasma concentration–time curve (AUC) of pravastatin. This result was supported by in vitro analysis showing that the intrinsic maximum velocity normalized by the expression level for *OATP1B1*15* variant was drastically reduced compared with *OATP1B1*1a*.[7–9] Subsequently, 2 clinical studies showed that the Val174Ala mutation also increased the AUC of pravastatin in white subjects.[11,12] Very recently, Niemi et al[13,14] reported that the pharmacokinetics of fexofenadine and repaglinide was also affected by the Val174Ala mutation. These results suggest that the Val174Ala mutation in OATP1B1 reduces the transport function. On the other hand, Mwinyi et al[12] showed that the AUC of pravastatin in subjects with **1a/*1b* (Asn130Asp) or **1b/*1b* alleles tended to be lower than that in **1a* homozygotes. However, they did not completely separate the subjects with the **1b* allele from those with the **1a* allele, and so we cannot directly compare the effect of the **1b* allele with that of the **1a* allele. The allele frequency of *OATP1B1*1b* was reported to be high and showed some ethnic differences (eg, 0.30 in white Americans [n = 49],[9] 0.74 in black Americans [n = 44],[9] and 0.63 in Japanese subjects [n = 120][10]), implying that this might cause the ethnic differences in the pharmacokinetics of drugs. Therefore we were particularly interested in the effect of the Asn130Asp variant of OATP1B1 on the pharmacokinetics of 3 drugs, pravastatin, valsartan, and temocapril, and we classified the subjects into 4 groups, **1a/*1a*, **1b/*1b*, **1a/*15*, and **1b/*15* carriers, to directly investigate the difference in the pharmacokinetics of the subjects with the **1a* and **1b* alleles (**1a/*1a* versus **1b/*1b* and **1a/*15* versus **1b/*15*).

Valsartan is a novel angiotensin II receptor antagonist, and temocapril is an angiotensin-converting enzyme inhibitor. Drugs in these categories are widely used for the treatment of hypertension. Valsartan is mainly eliminated via the liver. Valsartan itself is pharmacologically active and is thought to be excreted into the bile in unchanged form without extensive metabolism.[15] Because of its hydrophilicity and carboxyl moiety, some organic anion transporters may be involved in the hepatic clearance of valsartan. Temocapril is an esterified prodrug and is rapidly converted to the active metabolite temocaprilat by carboxyl esterase.[16] Temocaprilat is mainly excreted into the bile, whereas the active metabolites of other angiotensin-converting enzyme inhibitors such as enalaprilat are mainly excreted into the urine because temocaprilat, but not enalaprilat, can interact with multidrug resistance associated protein 2 (MRP2), which is an efflux transporter located on the apical membrane.[17] Sasaki et al[18] demonstrated that transcellular vectorial transport of temocaprilat was

observed in OATP1B1/MRP2 double-transfected cells, suggesting that temocaprilat is a substrate of OATP1B1.

Therefore the purpose of this study was to clarify the importance of the OATP1B1 haplotype, especially the **1b* allele, in the pharmacokinetics of the OATP1B1 substrates pravastatin, valsartan, and temocaprilat, as well as to determine whether the clearances of OATP1B1 substrate drugs in each subject are well correlated with one another in healthy Japanese volunteers.

METHODS

Subjects. Twenty-three healthy male Japanese volunteers participated in this clinical study. They were recruited from a population of 100 male Japanese volunteers whose OATP1B1 haplotype was prescreened after written informed consent was obtained. The genotyping method of OATP1B1 has been described previously.[10] The haplotypes of OATP1B1 in the 23 participants were **1a/*1a* (n = 5), **1a/*15* (n = 6), **1b/*1b* (n = 7), and **1b/*15* (n = 5). The participants were aged between 20 and 35 years. Each participant had a body weight of between 50 and 80 kg and a body mass index of between 17.6 and 26.4 kg/m^2. Within 1 month before this clinical study was started, a medical history was obtained from the participants, who then underwent a physical examination, electrocardiography, routine blood testing, and urinalysis. They were also screened for narcotic drugs and psychotropic substances. This allowed us to confirm that all of the subjects were able to participate in this study.

Study design. This study protocol was approved by the Ethics Review Boards at both the Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, and Kannondai Clinic, Tsukuba, Japan. All participants provided written informed consent. All subjects took part in the 3-period crossover trial and received pravastatin, valsartan, and temocapril in a random sequence. There was a washout period of 1 week between each administration. In each period subjects came to the clinic on the day before drug administration. After an overnight fast, each subject received 10 mg pravastatin sodium (Mevalotin tablet; Sankyo, Tokyo, Japan), 2 mg temocapril hydrochloride (Acecol tablet; Sankyo), or 40 mg valsartan (Diovan tablet; Novartis, Basel, Switzerland). Venous blood samples (7 mL each) were collected in tubes containing heparin before and at 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 12, and 24 hours after drug administration. Urine samples were collected for 24 hours. Plasma was separated by centrifugation. Plasma and urine samples were stored at −80°C until analysis. Alcohol, grapefruit juice, St John's wort, and other drugs were not permitted from 2 days before admission to the clinic until the end of the study periods, and smoking was prohibited during the study periods. During the study periods, standardized meals were served to all subjects at scheduled times. For the safety of subjects, after the end of each period, all subjects underwent a physical examination and routine blood testing and urinalysis were carried out.

Quantification of concentrations of pravastatin and its metabolite, RMS-416, in plasma and urine. Concentrations of pravastatin and RMS-416 in plasma and urine were measured by liquid chromatography–tandem mass spectrometry as described in an earlier report.[19] One milliliter of plasma was mixed with 100 μL internal standard (R-122798, 800 ng/mL; prepared by Sankyo), 1 mL 10% methanol, and 300 μL 0.5-mol/L phosphate buffer (pH 4.0). In addition, 0.5 mL urine was mixed with 50 μL internal standard (R-122798), 0.5 mL 10% methanol, and 300 μL 0.5-mol/L phosphate buffer (pH 4.0). The mixture was applied to a Bond Elut C8 cartridge (200 mg/3 mL) (Varian, Palo Alto, Calif), washed twice with 3 mL 5% methanol (plasma) or distilled water (urine), and eluted with 2 mL acetonitrile. The eluate was evaporated under nitrogen gas at 40°C, mixed with 120 μL acetonitrile, and ultrasonicated for 3 minutes. Then, 180 μL 10-mmol/L ammonium acetate was added, and aliquots (20 μL for plasma and 10 μL for urine) were injected into the liquid chromatography–tandem mass spectrometry system. Separation by HPLC was conducted with an Agilent 1100 Series system (Agilent Technologies, Palo Alto, Calif) with an Inertsil ODS-3 column (4.6 × 150 mm, 5 μm; GL Sciences, Tokyo, Japan). The composition of the mobile phase was acetonitrile/water/ammonium acetate/formic acid/triethylamine (400:600:0.77:0.2:0.6 [vol/vol/wt/vol/vol]). The flow rate was 1 mL/min. Mass spectra were determined with an API 4000 tandem mass spectrometer (MDS Sciex, Concord, Ontario, Canada) in the negative ion–detecting mode at the atmospheric pressure–chemical ionization interface. The turbo gas temperature was 600°C. The samples were ionized by reacting with solvent-reactant ions produced by the corona discharge (−5.0 μA) in the chemical ionization mode. The precursor ions of pravastatin at mass-to-charge ratio (*m/z*) 423.2, RMS-416 at *m/z* 423.2, and R-122798 at *m/z* 409.2 were admitted to the first quadrupole (Q1). After the collision-induced fragmentation in the second quadrupole (Q2), the product ions of pravastatin at *m/z* 321.1, RMS-416 at *m/z* 321.3, and R-122798 at *m/z* 321.4 were monitored in the third quadrupole (Q3). The peak area ratio of each compound to the corresponding

internal standard was calculated with Analyst Software (version 1.3.1; Applied Biosystems, Foster City, Calif). The calibration curves were linear over the standard concentration range of 0.1 ng/mL to 100 ng/mL for pravastatin and RMS-416 in plasma, 20 ng/mL to 2000 ng/mL for pravastatin in urine, and 5 ng/mL to 500 ng/mL for RMS-416 in urine.

Quantification of valsartan concentration in plasma and urine. One hundred microliters of plasma or urine was mixed with 100 μL internal standard ([$^2H_9$]-valsartan in 50% methanol, 500 ng/mL; prepared by Novartis Pharma, Basel, Switzerland) and 300 μL 2% trifluoroacetic acid (TFA) aqueous solution. The mixture was applied to a 96-well Empore Disk Plate C18 SD (Sumitomo 3M, Tokyo, Japan); washed 3 times with 200 μL 1% TFA aqueous solution, 1% TFA in 5% methanol, and 1% TFA in 20% methanol; and eluted twice with 100 μL methanol. The eluate was evaporated under nitrogen gas at 40°C, mixed with 100 μL (for plasma) or 400 μL (for urine) methanol/acetonitrile/0.1% TFA (35:20:45 [vol/vol/vol]), and ultrasonicated for 3 minutes. Then, 5-μL aliquots were injected into the liquid chromatography–tandem mass spectrometry system. Separation by HPLC was conducted with an Agilent 1100 Series system (Agilent Technologies) with a Symmetry C18 column (2.1 × 30 mm, 3.5 μm; Waters, Milford, Mass). The composition of the mobile phase was methanol/acetonitrile/0.1% TFA (35:20:45 [vol/vol/vol]). The flow rate was 0.2 mL/min. Mass spectra were determined with an API 4000 tandem mass spectrometer (Applied Biosystems) in the positive ion–detecting mode at the electrospray ionization interface. The turbo gas temperature was 500°C, and the spray voltage was 5500 V. The precursor ions of valsartan at *m/z* 436.1 and [$^2H_9$]-valsartan at *m/z* 445.1 were admitted to the first quadrupole (Q1). After the collision-induced fragmentation in the second quadrupole (Q2), the product ions of valsartan at *m/z* 291.1 and [$^2H_9$]-valsartan at *m/z* 300.1 were monitored in the third quadrupole (Q3). The peak area ratio of each compound to the corresponding internal standard was calculated with Analyst Software (version 1.3.1; Applied Biosystems). The calibration curves were linear over the standard concentration range of 2 ng/mL to 5000 ng/mL for plasma and 20 ng/mL to 5000 ng/mL for urine.

Quantification of temocapril and temocaprilat concentrations in plasma and urine. Two hundred microliters of plasma was mixed with 200 μL internal standard ([$^2H_5$]-temocaprilat, 10 ng/mL; prepared by Sankyo), 2 mL 0.1% formic acid, and 200 μL methanol. Then, 500 μL urine was mixed with 200 μL internal standard ([$^2H_5$]-temocaprilat), 500 μL 0.5% formic acid, and 500 μL methanol. The mixture was applied to a Sep-Pak Vac PS-2 cartridge (200 mg/3 mL) (Waters), washed with twice with 3 mL distilled water, and eluted twice with 3 mL methanol. The eluate was evaporated under nitrogen gas at 45°C, mixed with 280 μL methanol, and ultrasonicated for 3 minutes. Then, 120 μL 0.2% acetic acid was added, and 10-μL aliquots were injected into the liquid chromatography–tandem mass spectrometry system. Separation by HPLC was conducted with an Agilent 1100 Series system (Agilent Technologies) with a Symmetry C18 column (2.1 × 150 mm, 5 μm; Waters). The composition of the mobile phase was methanol/water/acetic acid (700:300:2 [vol/vol/vol]). The flow rate was 0.2 mL/min. Mass spectra were determined with an API 4000 tandem mass spectrometer (Applied Biosystems) in the positive ion–detecting mode at the electrospray ionization interface. The turbo gas temperature was 600°C, and the spray voltage was 5500 V. The precursor ions of temocapril at *m/z* 477.0, temocaprilat at *m/z* 448.9, and [$^2H_5$]-temocaprilat at *m/z* 454.0 were admitted to the first quadrupole (Q1). After the collision-induced fragmentation in the second quadrupole (Q2), the product ions of temocapril at *m/z* 270.0, temocaprilat at *m/z* 269.8, and [$^2H_5$]-temocaprilat at *m/z* 269.9 were monitored in the third quadrupole (Q3). The peak area ratio of each compound to the corresponding internal standard was calculated with Analyst Software (version 1.3.1; Applied Biosystems). The calibration curves were linear over the standard concentration range of 0.5 ng/mL to 200 ng/mL for temocapril and temocaprilat in plasma, 1 ng/mL to 80 ng/mL for temocapril in urine, and 5 ng/mL to 400 ng/mL for temocaprilat in urine.

Uptake study by use of OATP1B1 expression system. The OATP1B1-expressing human embryonic kidney (HEK) 293 cells and vector-transfected control cells have been established previously, and the transport study was carried out as described previously.[5] Tritium-labeled valsartan and unlabeled valsartan were kindly donated by Novartis Pharma, and carbon 14–labeled temocaprilat and unlabeled temocaprilat were donated by Sankyo. Uptake was initiated by the addition of Krebs-Henseleit buffer containing radiolabeled and unlabeled substrates after cells had been washed twice and preincubated with Krebs-Henseleit buffer at 37°C for 15 minutes. The Krebs-Henseleit buffer consisted of 118-mmol/L sodium chloride, 23.8-mmol/L sodium bicarbonate, 4.8-mmol/L potassium chloride, 1.0-mmol/L potassium phosphate [monobasic], 1.2-mmol/L magnesium sulfate, 12.5-mmol/L *N*-[2-hydroxyethyl]piperazine-*N*′-

[2-ethanesulfonic acid] (HEPES), 5.0-mmol/L glucose, and 1.5-mmol/L calcium chloride adjusted to pH 7.4. The uptake was terminated at a designated time by the addition of ice-cold Krebs-Henseleit buffer after removal of the incubation buffer. Cells were then washed twice with 1 mL of ice-cold Krebs-Henseleit buffer, solubilized in 500 μL of 0.2N sodium hydroxide, and kept overnight at 4°C. Aliquots (500 μL) were transferred to scintillation vials after the addition of 250 μL of 0.4N hydrochloric acid. The radioactivity associated with the cells and incubation buffer was measured in a liquid scintillation counter (LS6000SE; Beckman Coulter, Fullerton, Calif) after the addition of 2 mL of scintillation fluid (Clear-sol I; Nacalai Tesque, Kyoto, Japan) to the scintillation vials. The remaining 50 μL of cell lysate was used to determine the protein concentration by the method of Lowry et al[18a] with bovine serum albumin as a standard.

Transcellular transport study by use of double-transfected cells. The transcellular transport study was performed as reported previously by Sasaki et al.[18] In brief, Madin-Darby canine kidney II (MDCKII) cells were grown on Transwell membrane inserts (6.5-mm diameter, 0.4-μm pore size; Corning Coster, Bodenheim, Germany) at confluence for 3 days, and the expression level of transporters was induced with 5-mmol/L sodium butyrate for 2 days before the transport study. Cells were first washed with Krebs-Henseleit buffer at 37°C. Subsequently, substrates were added in Krebs-Henseleit buffer either to the apical compartments (250 μL) or to the basolateral compartments (1 mL). After a designated period, the aliquot of the incubation buffer in the opposite compartments (100 μL from apical compartment or 250 μL from basal compartment) was collected. The amount of tritium-labeled estradiol-17β-glucuronide in the samples was determined by a liquid scintillation counter (LS6000SE; Beckman Coulter), and the amount of temocapril and RMS-416 in the samples was determined by liquid chromatography–mass spectrometry as described later.

Quantification of temocapril concentration in Krebs-Henseleit buffer. A 50-μL sample was mixed vigorously with 250 μL of ethyl acetate. Two hundred microliters of supernatant was collected, dried up by a centrifugal concentrator (TOMY, Tokyo, Japan), and dissolved in 40 μL dimethylsulfoxide. Thirty-microliter aliquots were injected into the liquid chromatography–tandem mass spectrometry system. Separation by HPLC was conducted with a Waters Alliance 2695 Separations Module with an L-column octadecylsilane (2.1 × 150 mm, 5 μm; Chemicals Evaluation and Research Institute, Tokyo, Japan). The composition of the mobile phase was acetonitrile/0.05% formic acid (40:60 [vol/vol]). The flow rate was 0.3 mL/min. Mass spectra were determined with a Micromass ZQ2000 mass spectrometer (Waters) in the positive ion–detecting mode at the electrospray ionization interface. The source temperature and desolvation temperature were 100°C and 350°C, respectively. The capillary, cone, and extractor voltages were 3200 V, 30 V, and 5 V, respectively. The cone gas flow and desolvation gas flow were 65 L/h and 375 L/h, respectively. The mass spectrometer was operated in the selected ion monitoring mode by use of a positive ion, *m/z* 477.30 for temocapril. The retention time of temocapril was approximately 3.7 minutes. Standard curves were linear over the range of 3 to 300 nmol/L.

Quantification of RMS-416 concentration in Krebs-Henseleit buffer. A 60-μL sample was mixed vigorously with 60 μL of methanol including internal standard (0.5 μg/mL R-122798; kindly donated by Sankyo) and deproteinized by centrifugation for 10 minutes at 15,000 rpm at 4°C. Then, 50 μL of supernatant was injected into the liquid chromatography–tandem mass spectrometry system. Separation by HPLC was conducted with a Waters Alliance 2695 Separations Module with an Inertsil ODS-3 column (4.6 × 150 mm, 5 μm; GL Sciences). The composition of the mobile phase was acetonitrile/ammonium acetate, 10 mmol/L (pH 4) (40:60 [vol/vol]). The flow rate was 0.3 mL/min. Mass spectra were determined with a Micromass ZQ2000 mass spectrometer (Waters) in the negative ion–detecting mode at the electrospray ionization interface. The source temperature and desolvation temperature were 100°C and 350°C, respectively. The capillary, cone, and extractor voltages were 3200 V, 20 V and 5 V, respectively. The cone gas flow and desolvation gas flow were 65 L/h and 375 L/h, respectively. The mass spectrometer was operated in the selected ion monitoring mode by use of respective positive ions, *m/z* 423.30 for RMS-416 and *m/z* 409.30 for R-122798 (internal standard). The retention time of RMS-416 and R-122798 was approximately 3.6 minutes and 2.6 minutes, respectively. Standard curves were linear over the range of 5 to 1000 nmol/L.

Pharmacokinetic and statistical analyses. The AUC from time 0 to 24 hours (AUC_{0-24}) was calculated by the linear trapezoidal rule. Renal clearance (CL_r) was calculated by division of the cumulative amount of drug in urine collected for 24 hours by AUC_{0-24}. All pharmacokinetic data are given as mean ± SD. Statistical differences between the data for each haplotype group were determined by ANOVA, followed by the Fisher

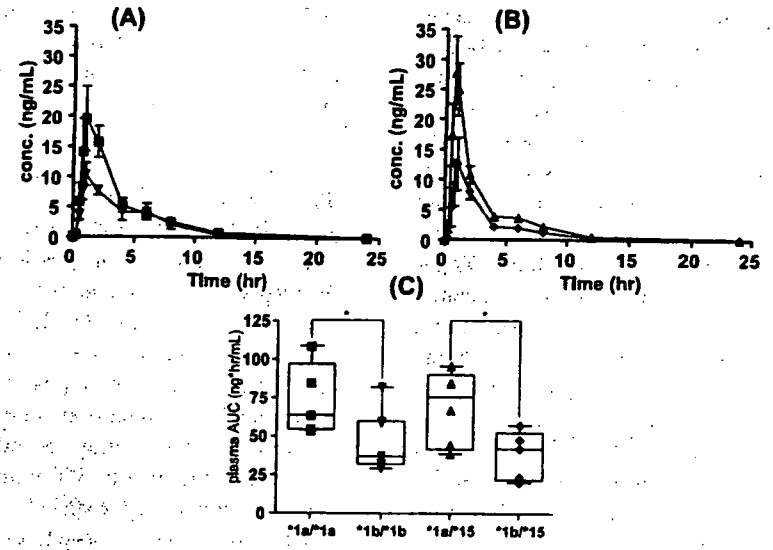

Fig 1. Effect of organic anion transporting polypeptide (OATP) 1B1 haplotype on pharmacokinetics of pravastatin. Plasma concentration (conc)–time profiles of pravastatin after oral administration of 10 mg pravastatin in *OATP1B1*1a/*1a* subjects (*squares*, n = 5) and **1b/*1b* subjects (*inverted triangles*, n = 7) (A) and in **1a/*15* subjects (*triangles*, n = 6) and **1b/*15* subjects (*diamonds*, n = 5) (B). Each *point* represents mean ± SD. C, Box-whisker plot of area under plasma concentration–time curve (AUC) of pravastatin in each haplotype group. The *horizontal line* within each *box* represents the median. The *box edges* represent the lower (25th) and upper (75th) quartiles. The *whiskers* extend from the lower and upper quartiles to the furthest data points still within a distance of 1.5 interquartile ranges from the lower and upper quartiles. Individual data points were overlaid on the box-whisker plot. *Asterisk*, Statistically significant difference shown by ANOVA with Fisher least significant difference test ($P < .05$).

least significant difference test. $P < .05$ was considered to be statistically significant.

RESULTS

Effect of OATP1B1 haplotype on pharmacokinetics of pravastatin and its metabolite, RMS-416. After oral administration of pravastatin, the plasma concentration of pravastatin in *OATP1B1*1b/*1b* subjects was lower than that in **1a/*1a* subjects (Fig 1, *A*). Similarly, the plasma concentration in **1b/*15* subjects was lower than that in **1a/*15* subjects (Fig 1, *B*). The mean $AUC_{0\text{-}24}$ of pravastatin in **1b/*1b* subjects was significantly lower than that in **1a/*1a* subjects (65% of **1a/*1a*), and the $AUC_{0\text{-}24}$ in **1b/*15* subjects was significantly lower than that in **1a/*15* subjects (55% of **1a/*15*) (Fig 1, *C*, and Table I). In addition, CL_r was not significantly different among the haplotype groups (Table I). Pravastatin was converted to RMS-416 by chemical epimerization. We also calculated the concentration of the sum of pravastatin and RMS-416 in plasma and urine. The $AUC_{0\text{-}24}$ value of the sum of pravastatin and RMS-416 in **1b* carriers tended to be lower than that in **1a* carriers, whereas this value in **15* carriers tended to be higher than that in non-**15* carriers (Table I). The CL_r calculated from the sum of pravastatin and RMS-416 was not markedly different between each haplotype group.

Effect of OATP1B1 haplotype on pharmacokinetics of valsartan. After oral administration of valsartan, the plasma concentration of valsartan in *OATP1B1*1b/*1b* subjects was lower than that in **1a/*1a* subjects (Fig 2, *A*) and the plasma concentration in **1b/*15* subjects was lower than that in **1a/*15* subjects (Fig 2, *B*). Although the difference did not reach statistical significance, the mean $AUC_{0\text{-}24}$ of valsartan in **1b/*1b* subjects tended to be lower than that in **1a/*1a* subjects (73% of **1a/*1a*), and the $AUC_{0\text{-}24}$ in **1b/*15* subjects was significantly lower than that in **1a/*15* subjects (67% of **1a/*15*) (Fig 2, *C*, and Table I), exhibiting a trend similar to pravastatin. The CL_r was almost the same in each haplotype group (Table I).