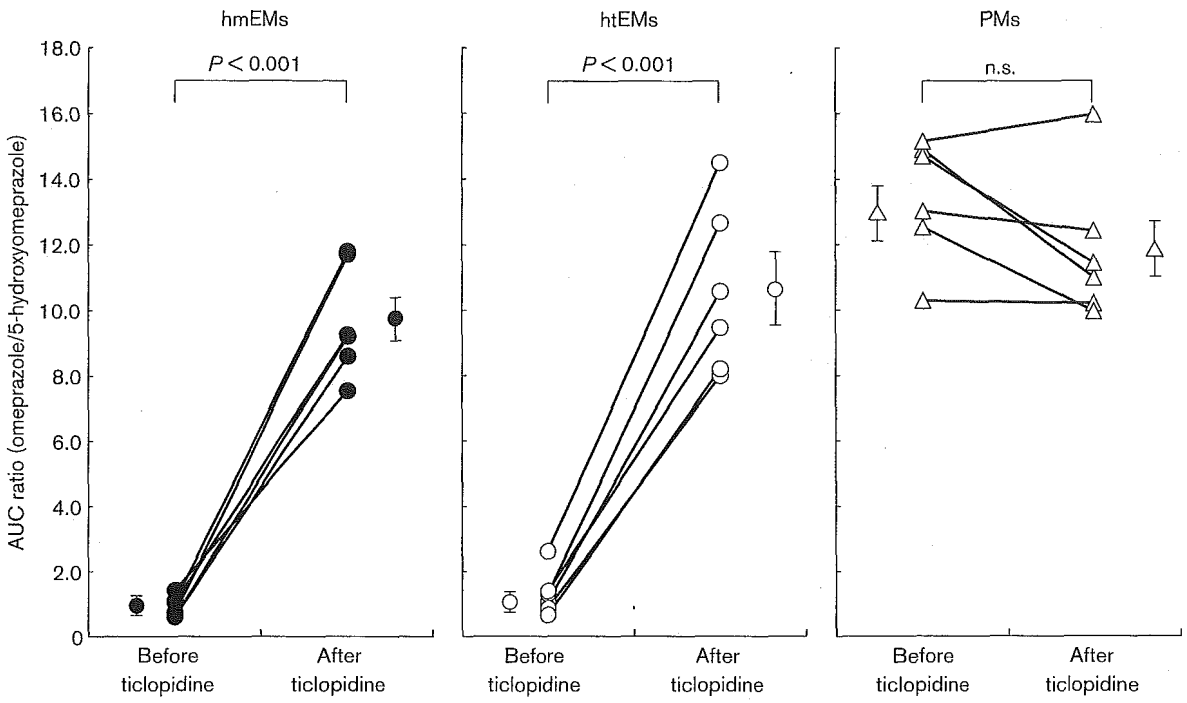
Fig. 3



CYP2C19 genotype-dependent changes in the AUC ratio (omeprazole/5-hydroxyomeprazole) by the repeated 8-day ticlopidine administration.

Table 2 **Pharmacokinetic parameters of ticlopidine following the first (day 1) and seventh (day 7) doses in relation to *CYP2C19* genotypic status**

	Homozygous EMs ($n=6$)	Heterozygous EMs ($n=6$)	PMs ($n=6$)
Day 1			
C_{max} (ng/ml)	496.5 ± 70.3	642.6 ± 146.3	513.9 ± 96.5
T_{max} (h)	2.2 ± 0.5	1.5 ± 0.2	1.5 ± 0.2
$T_{1/2}$ (h)	10.3 ± 1.1	12.7 ± 2.4	13.3 ± 1.4
AUC_{0-24} (ng·h/ml)	1698.5 ± 259.5	1847.3 ± 289.1	1426.6 ± 347.9
Day 7			
C_{max} (ng/ml)	1059.8 ± 123.7**	816.4 ± 87.5	614.4 ± 166.8
T_{max} (h)	1.8 ± 0.2	2.0 ± 0.4	1.7 ± 0.5
$T_{1/2}$ (h)	12.9 ± 0.8	17.3 ± 5.7	15.8 ± 3.0
AUC_{0-24} (ng·h/ml)	3933.3 ± 503.6**	3346.2 ± 279.7*	2506.9 ± 800.9*
Ratio of day 7 to day 1 data			
C_{max} (ng/ml)	2.3 ± 0.3	1.7 ± 0.6	1.1 ± 0.1
T_{max} (h)	1.0 ± 0.2	1.6 ± 0.4	1.2 ± 0.3
$T_{1/2}$ (h)	1.3 ± 0.1	1.3 ± 0.2	1.2 ± 0.3
AUC_{0-24} (ng·h/ml)	2.4 ± 0.2	2.0 ± 0.3	1.7 ± 0.1

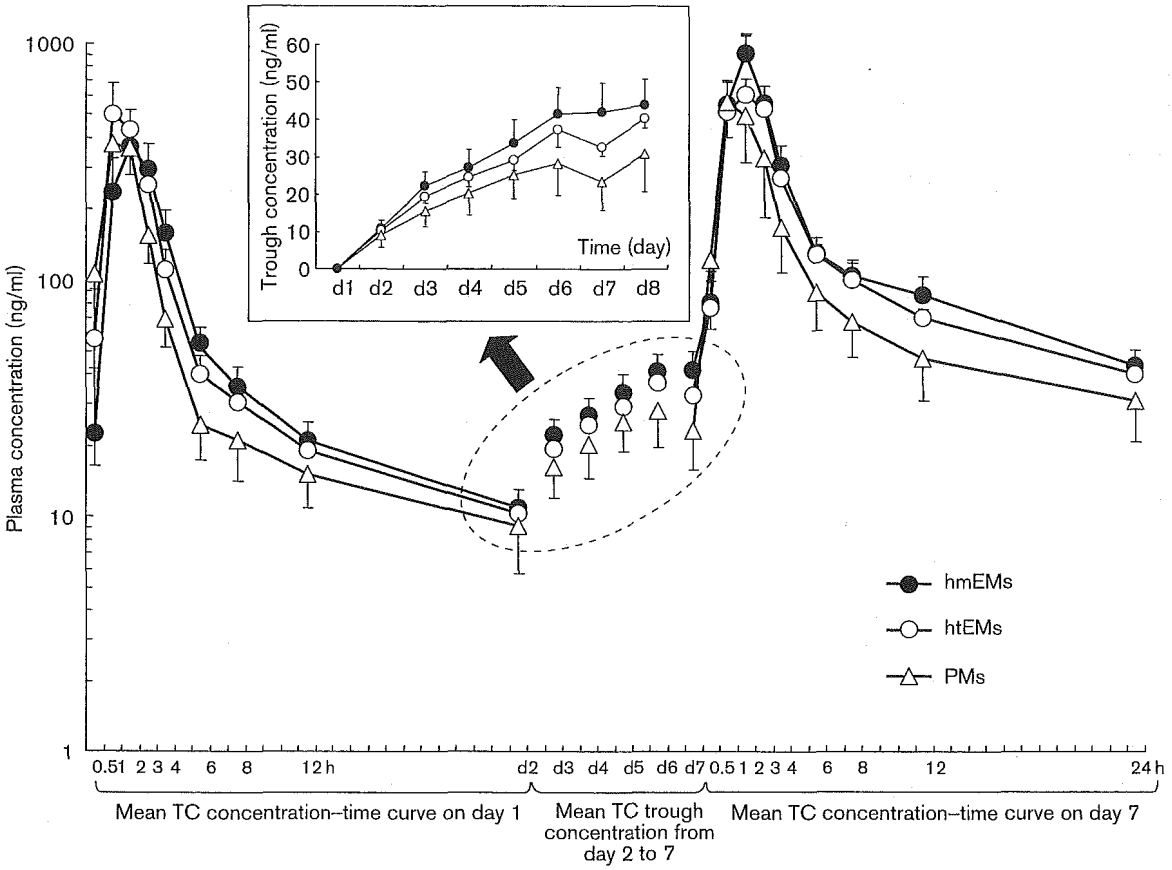
*$P<0.01$ (versus day 1 data); **$P<0.001$ (versus day 1 data).

platelet aggregation. This relationship appeared to exist regardless of *CYP2C19* genotypic status.

Discussion

The primary objective of this study was to evaluate the pharmacokinetics and pharmacodynamics of ticlopidine in relation to genetically determined CYP2C19 polymorphism. The most important findings were that: (1) a significant intergenotypic change in CYP2C19 activity (i.e., omeprazole 5-hydroxylation capability) was observed after multiple doses of ticlopidine; (2) although statistically significant intergenotypic differences in the pharmacokinetic parameters of ticlopidine were not observed, the accumulation ratio of ticlopidine [i.e., the AUC_{0-24} ratio of day 7 to day 1] and trough concentrations at all observed points tended to be greater in the hmEM subjects; and (3) the mean percentage inhibition of

Fig. 4



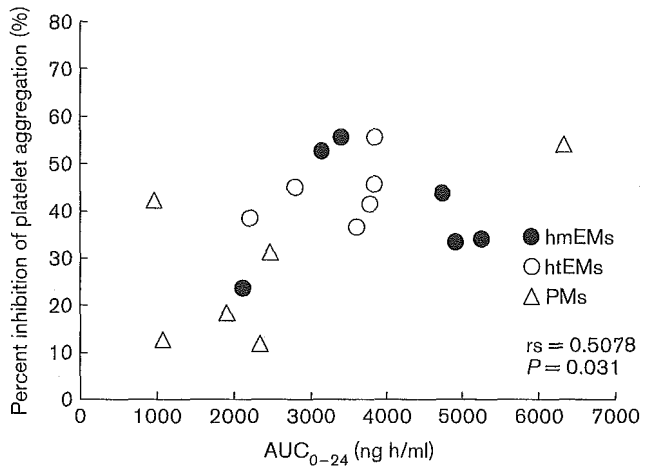
Mean plasma concentrations of ticlopidine (TC) in relation to *CYP2C19* genotypic status during the study period. The mean trough concentrations of ticlopidine from day 2 to day 8 are also indicated in the inset.

ADP-induced platelet aggregation increased with the repeated doses, but irrespective of CYP2C19 genotypic status. These results indicate that ticlopidine is a potent inhibitor *in vivo* and a less prominent substrate for CYP2C19 and that the *CYP2C19* polymorphic genes are likely important in terms of the interaction profiles of ticlopidine, but not in terms of platelet aggregation.

This study describes a substantial decrease in the CYP2C19 activity after repeated doses of ticlopidine in both the groups of EMs, but not in PM subjects. Our results confirm previous human studies showing that ticlopidine is a potent inhibitor of CYP2C19 [18,21]. Importantly, the inhibition of the CYP2C19 activity occurred only in the EM subjects, in whom hepatic CYP2C19 is believed to be sufficiently expressed. Similar genotype-dependent interactions have been reported for certain drug combinations (CYP2C19 substrate plus inhibitors) such as omeprazole plus moclobemide [22] and fluvoxamine plus chloroguanide [23]. In all cases, the pharmacokinetic parameters of substrates for CYP2C19 and their metabolites in the EM subjects changed to the values similar to those in the PM subjects. Thus, multiple doses of ticlopidine may be associated with a change in phenotypic status from extensive to poor metabolizers, so-called 'phenocopying' [24,25], when substrates for CYP2C19 are co-administered to EM subjects.

Despite a long history of clinical use, there is limited published information about the pharmacokinetics of ticlopidine. As shown in Fig. 4, the mean trough concentrations continued to rise over the seventh day of dosing, suggesting that an accumulation or saturation in the metabolism may occur during repeated dosings. These results are well consistent with the earlier reports that plasma ticlopidine concentrations (e.g., C_{max} and AUC) increase by three- to four-fold on repeated twice-daily dosings over 2 to 3 weeks [10,11]. In the present study, there was a trend toward the observation that the accumulation of ticlopidine seemed to be influenced by *CYP2C19* polymorphism: the accumulation ratio tended to be greater in the hmEM subjects. Similar to the ratio, the mean trough levels also tended to be higher in the hmEM subjects compared with the PM subjects, and

Fig. 5



Correlation between individual % inhibition of platelet aggregation and the AUC_{0-24} of ticlopidine on the 7th day of dosing. Individual *CYP2C19* genotypes are also indicated.

the htEM subjects had the values between those in hmEM and PM subjects throughout the study period. Ticlopidine is rapidly oxidized by recombinant CYP2C19 with the formation of two major metabolites, the keto tautomer of 2-hydroxyticlopidine and dimers of ticlopidine *S*-oxide [16]. The former has actually been detected as a metabolite of ticlopidine *in vivo* [26]. Recently, Ha-Duong *et al.* [16] demonstrated that this CYP2C19-catalyzed oxidation of ticlopidine occurs in parallel with an inactivation of CYP2C19. Since the ticlopidine-mediated inactivation of CYP2C19 is due to the covalent binding of the reactive ticlopidine *S*-oxide to the CYP2C19 active site, accumulation or saturation would be expected to more discernibly occur in the EM subjects, especially in the hmEM subjects. However, in addition to CYP2C19, other CYP enzymes, such as CYP2D6, CYP3A4 [16,19] and CYP2B6 (our unpublished microsomal experiments), may be involved in the oxidation of ticlopidine. Furthermore, the current in-vitro studies examined ticlopidine to undergo the principal routes of metabolism (i.e., *N*-dealkylation, *N*-oxidation and oxidation of the thiophene ring) by peroxidases and monoamine exidase [19]. Thus, this multiple enzyme-mediated metabolism may explain why there were no significant differences in the accumulation ratio among the three *CYP2C19* genotypic groups.

In this study we used the AUC_{0-24} of ticlopidine on day 7 for the calculation of the accumulation index. For a better understanding of the potential effects of genetic variation on the pharmacokinetics of ticlopidine, sufficient time to reach the steady state (i.e., 14 days) and long duration of sampling time (i.e., 96 h) is needed. For these limitations, the pharmacokinetics data on ticlopidine presented here should be viewed as the apparent values obtained during the pseudo-steady state. Furthermore, whether and to what extent CYP2C19 would be involved in the overall metabolism of ticlopidine remain unanswered from this in-vivo human study.

Despite its clinical usefulness, chronic administration of ticlopidine results in a relatively high incidence of hepatotoxicity [27–29]. Although the mechanisms involved in the ticlopidine-induced hepatic injury remain unknown, immune mechanisms and drug hypersensitivity have been proposed. Previous studies indicated that tienilic acid (a thiophene derivative and a substrate of CYP2C9) and ticlopidine act as a selective suicide substrate of CYP2C9 [17] and CYP2C19 [16], respectively. It is well known that tienilic acid sometimes induces immunoallergic hepatitis in a subset of patients who produce anti-liver–kidney microsome antibody type 2 (LKM-2) autoantibodies [30–33]. Since LKM autoantibodies are observed in autoimmune hepatitis, in some patients with drug-induced hepatitis, they are markers of autoimmune hepatitis. In contrast to LKM-2 antibodies, LKM-1 antibodies mostly target CYP2D6 [34]. In this regard, we monitored LKM-1 and LKM-2 in the present study. However, no changes in these antibody markers were observed during the present short study period (data not shown).

Although a significant positive correlation was observed between the individual percent inhibition of platelet aggregation and the AUC_{0-24} of ticlopidine on the seventh day of dosing, a large interindividual difference in platelet aggregation could not be explained by the CYP2C19 polymorphism. Antiaggregant effects were noted at 24 to 48 h with a maximal effect observed after 3 to 5 days of dosings [35,36] or after 6 to 10 days in a Chinese study [37]. Thus, the AUC_{0-24} observed on day 7 may be as a pharmacokinetic parameter obtained at the appropriate time of the maximal antiaggregant effect. Ticlopidine does not inhibit ADP-induced platelet aggregation *in vitro*, and hepatic conversion into active metabolite(s) is required for drug action [38]. More than thirteen metabolites have been described to date, but those responsible for the antiplatelet effect have not yet been well documented. Very recently, Yoneda *et al.* [39] identified a metabolite with a potent antiplatelet activity, UR-4501, which was generated after incubation of 2-oxo-ticlopidine with phenobarbital-induced rat liver homogenate. They indicated that UR-4501 produced a concentration-dependent inhibition of ADP-induced human platelet aggregation, whereas 2-oxo-ticlopidine did not elicit inhibitory responses. Although the major CYP enzyme(s) responsible for the formation of UR-4501 has yet to be identified, our results suggest that it is not CYP2C19. An in-vitro study is definitely required for screening CYP enzyme(s) responsible for the formation of the pharmacologically active metabolite(s) such as UR-4501.

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Different contributions of polymorphisms in *VKORC1* and *CYP2C9* to intra- and inter-population differences in maintenance dose of warfarin in Japanese, Caucasians and African-Americans

Harumi Takahashi[a], Grant R. Wilkinson[b], Edith A. Nutescu[c], Takashi Morita[d], Marylyn D. Ritchie[e], Maria G. Scordo[f], Vittorio Pengo[g], Martina Barban[g], Roberto Padrini[h], Ichiro Ieiri[i], Kenji Otsubo[i], Toshitaka Kashima[j], Sosuke Kimura[j], Shinichi Kijima[a] and Hirotoshi Echizen[a]

Objective To investigate pharmacokinetic and pharmacodynamic factors associated with population differences in warfarin doses needed to achieve anticoagulation, in particular the possible involvement of genetic variability in vitamin K epoxide reductase (VKOR) and CYP2C9.

Methods Warfarin maintenance dose, unbound plasma S-warfarin concentration [Cu(S)] and INR were determined in 157 Caucasians, 172 Japanese, and 36 African-Americans stably anticoagulated patients. In a subset ($n=166$), fully carboxylated plasma normal prothrombin levels (NPT) were also measured. Genotyping for seven *CYP2C9* (*CYP2C9*1* through *6* and **11*) and seven *VKORC1* variants were performed in 115 Caucasians and 64 Japanese patients and 66 healthy African-Americans. Multivariate analysis was performed to identify covariates associated with warfarin requirement.

Results The relationship between NPT and Cu(S) indicated Japanese are more susceptible to inhibition of NPT production by S-warfarin than the other two populations. *VKORC1* 1173 C>T had a greater frequency in Japanese (89.1%) than Caucasians (42.2%) and African-Americans (8.6%). *CYP2C9* variants with reduced metabolizing ability were less frequent in Japanese compared to the other two populations. The median warfarin dose was significantly higher in Caucasians than Japanese patients (5.5 versus 3.5 mg/day), however, when matched for *CYP2C9*1* homozygosity, no difference in dose was observed between *VKORC1* genotype-matched groups. Furthermore, *VKORC1* 1173C>T and *CYP2C9* (*2/*3/*11) genotypes, age and weight were identified as independent covariates contributing to interpatient variability in warfarin dosage.

Conclusions Both *VKORC1* and *CYP2C9* polymorphisms contribute to inter-population difference in warfarin doses among the three populations, but their contribution to intra-population variability may differ within each population. *Pharmacogenetics and Genomics* 16:101–110

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Keywords: warfarin, Japanese, Caucasian, African-Americans, polymorphism, VKORC1, CYP2C9

[a]Departments of Pharmacotherapy, [d]Biochemistry, Meiji Pharmaceutical University, Tokyo, Japan, [b]Department of Pharmacology, [e]Center for Human Genetics Research, Vanderbilt University School of Medicine, Nashville, Tennessee, USA, [c]Department of Pharmacy Practice, University of Illinois at Chicago, College of Pharmacy, Chicago, Illinois, USA, [f]Department of Clinical and Experimental Medicine and Pharmacology, University of Messina, Messina, Italy, [g]Departments of Clinical and Experimental Medicine, [h]Pharmacology and Anesthesiology, University of Padova, Padova, Italy, [i]Department of Hospital Pharmacy, Faculty of Medicine, Tottori University, Tottori, Japan and [j]Department of Cardiovascular Surgery, International Medical Center of Japan, Tokyo, Japan

Correspondence and requests for reprints to Dr Harumi Takahashi, Department of Pharmacotherapy, Meiji Pharmaceutical University, Noshio 2-522-1, Kiyose, Tokyo 204–8588, Japan
Tel/fax: +81-424-95-8453/8612; e-mail: harumit@my-pharm.ac.jp

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Introduction

Warfarin is the mainstay of anticoagulation therapy, worldwide. Its clinical use, however, is complicated by the fact that it has a narrow therapeutic index with associated adverse effects that are potentially serious, i.e., bleeding, and the dosage requirement to produce a required degree of anticoagulation varies widely between patients. The reason for the latter is multifactorial and includes determinants such as age [1–3], diet [4], and race [5–10]. Additionally, genetic factors determining the activity of CYP2C9 have been recently demonstrated to be important. This cytochrome P450 is largely

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responsible for the metabolism of S-warfarin, which is the enantiomer predominantly responsible for the drug's anticoagulant activity [11] – warfarin is administered as a racemate. In particular, two structural variants, CYP2C9.2 and CYP2C9.3, have greatly reduced catalytic activity compared to the wild-type enzyme, CYP2C9.1 [9,12], and retrospective studies have shown associations between the various genotypes and warfarin dose requirement and adverse effects [1–3,9,12–14]. It is apparent, however, that other factors, also possibly genetic, are important because, even when matched according to *CYP2C9* genotype, the dosing requirements for a similar degree of anticoagulation varies across populations and appear to be related to racial ancestry. For example, patients of Asian descent (Chinese [5,8,10], Japanese [9] and Malay [8,10]) require a lower maintenance dose of warfarin than Caucasians and Indians; by contrast, a higher dose is needed in African-Americans [6,7].

Warfarin's anticoagulant activity results from inhibition of hepatic vitamin K epoxide reductase (VKOR) that affects the synthesis of various coagulation factors. Recently, variants of the vitamin K epoxide reductase complex subunit 1 gene (*VKORC1*) have been described to have potentially functional consequences [15–21]. For instance, Rieder *et al.* [21] identified five major haplotypes (H1, H2, H7, H8 and H9) based upon 10 common single nucleotide polymorphisms (SNPs) of *VKORC1* in Caucasian and Asian populations and found that those having either H1 or H2 haplotypes required significantly lower dose of warfarin than those having H7, H8 or H9. In addition, these *VKORC1* haplotypes were correlated with the level of expression of mRNA of *VKORC1* in human liver.

Collectively, genetic polymorphisms involved in both pharmacokinetic (*CYP2C9*) and pharmacodynamic (*VKORC1*) factors, therefore, appear to interplay in the overall interindividual variability of warfarin doses; moreover, the contribution of each factor may differ among different ethnic populations. In this context, we initially studied the pharmacokinetics and pharmacodynamics of warfarin separately in a large number of patients having different ethnic backgrounds to assess population difference in the pharmacokinetic and pharmacodynamic phenotypes of warfarin among Caucasians, Japanese and African-Americans. We then examined the contribution of genetic polymorphisms of *CYP2C9* and *VKORC1* in smaller subsets of patients in order to study whether differences in the frequencies of *CYP2C9* and *VKORC1* variants would provide a possible explanation for the difference in warfarin requirements between these populations after taking other clinical covariates (e.g., demographics) into account.

Methods

Patients

Three hundred and sixty-five patients (157 Caucasians, 172 Japanese and 36 African-Americans) participated in the present study. The majority of them (140 Caucasians and 90 Japanese had been previously investigated with regard to S-warfarin metabolism [9,12]. Further analysis was performed in 179 patients in whom genetic information was available for both *CYP2C9* and *VKORC1*. Each patient received warfarin orally once daily for at least one month with the dose being titrated to an international nomalized ratio (INR) target value of 2.0 to 3.0 for Caucasian and African-Americans [22] and 1.5 to 2.5 for Japanese patients [23]. Clinical indications for anticoagulant therapy were prevention or treatment of thromboembolic disease (e.g., atrial fibrillation, deep vein thrombosis, or prosthetic valve replacement). Standard clinical laboratory tests indicated that all of the patients had normal liver function but three had impaired renal function (creatinine clearance ranging from 12 to 23 ml/min). Concurrent medications with potential to affect S-warfarin's metabolism included amiodarone ($n = 4$), NSAIDs ($n = 3$),, cimetidine ($n = 2$), thyroid hormone ($n = 6$) and carbamazepine ($n = 1$).

Study protocol

Blood (5–10 ml) was obtained 12 to 16 h after administration of the last dose of warfarin, during a routine clinic visit. Separated plasma was stored at −70°C until analyzed whereas the buffy coat was maintained at 4°C until extracted for DNA. The study protocol was approved by the IRBs of the respective institutions and written informed consent was obtained from each patient.

Pharmacokinetics and pharmacodynamics of warfarin

The plasma concentrations of warfarin's enantiomers were determined by a chiral high-pressure liquid chromatography-based method as previously described [24]. The extent of plasma protein binding was measured using ultrafiltration [24], which permitted estimation of the steady-state unbound plasma concentration [Cu(S)] and unbound oral clearance of S-warfarin [CLpo,u(S)] [9,25].

In addition to the INR value, warfarin's anticoagulant effect was also assessed in 166 patients (54 Caucasians, 91 Japanese and 21 African-Americans) through measurement of the plasma concentration of fully carboxylated or normal prothrombin (NPT) by the carinactivase-1 method [26]. A 'warfarin sensitivity index' [INR/Cu(S)] was also estimated for all patients.

VKORC1 and *CYP2C9* genotyping

DNA was extracted from the buffy coat of blood using a commercially available kit (Qiagen, Tokyo, Japan). Genotyping for variants in all coding regions and intron/exon boundaries of *VKORC1* (GenBank accession number AY587020) was performed by PCR and direct sequencing

using described primers to identify *VKORC1* 129C > T, 497T > G, 1173C > T, 1196G > A, 1331G > A, 3462C > T and 3730G > A [15,16,21]. In the present study, the position of a nucleotide was numbered according to a previously described system [16]: the A of the ATG initiation codon of AY587020 being denoted as position 1. Thus, the positions of 381, 3673, 6484, 6853 and 7566 of the reference sequence (AY587020) correspond to −4931, −1639, 1173, 1542 and 2255, respectively. Allelic variants of *CYP2C9* (*CYP2C9*1* through *CYP2C9*6*, and *CYP2C9*11*) were determined by either RFLP analysis or direct sequencing [9,27].

Genotypes for both *VKORC1* and *CYP2C9* were available for 179 patients (115 Caucasians and 64 Japanese). Because no DNA samples were available from African-American patients on warfarin, blood was commercially obtained from 64 healthy African-American subjects (ProMedDx, LLC, Norton, Massachusetts, USA) for analysis of the frequencies of the two gene's allelic variants. The patient haplotypes and their frequencies were estimated by PowerMarker (Ver. 3.23) and a haplotype association test was performed according to the method of Rieder *et al.* [21], which allowed classification of each patient into either Group A (comprising either H1 or H2 haplotypes) or Group B (comprising either H7, H8 or H9 haplotypes). Because the nucleotide at position 861 according to the Rieder's system was not examined, patients with the H7 haplotype were not distinguishable from those with an H8 haplotype. However, this did not affect classification of such individuals into Group B. A log-transformed maintenance dose adjusted for age, sex, body weight and *CYP2C9* genotype and warfarin sensitivity index [INR/Cu(S)] were compared between the patient groups with different haplotypes.

Statistics

Multiple comparisons between the mean values for the pharmacokinetic, pharmacodynamic and demographic data obtained from three populations were performed by ANOVA followed by the Tukey–Kramer test. Relationship between Cu(S) and INR in patients with different *VKORC1* (1173C > T) genotypes was examined by the Pearson's correlation test. Genetic data for deviation from the Hardy–Weinberg proportions were tested using the chi-square test. Multiple comparisons for allelic frequencies of *VKORC1* and *CYP2C9* variants between Caucasian, Japanese and African-American patients were performed by the chi-square test followed by the Tukey–Kramer test. Spearman's rank correlation test followed by the stepwise multiple regression analysis were performed to assess the contribution of patients' covariates [i.e., age, sex, body weight, racial ancestry (Caucasian versus Japanese) and genotypes (wild-type versus heterozygote versus homo- or the combined homozygote) of *VKORC1* and *CYP2C9*] to the overall variability of maintenance doses of warfarin. Squares of the adjusted correlation coefficient (r^2) and Akaike's Information Criterion (AIC) were employed to evaluate the goodness of model fitting. Data are presented as means ± SD or medians and the upper and lower quartile ranges (25 and 75 percentiles) where appropriate. A *P*-value of less than 0.05 was considered statistically significant for all analyses.

Results

The Caucasian patients were slightly older than the other two populations and there were also differences in body weight between the groups (Table 1). The daily maintenance dose of warfarin and its associated unbound concentration of the S-enantiomer were higher in African-Americans than in Caucasians who, in turn, had larger values than the Japanese; the reverse ranking was present

Table 1 Demographic characteristics of study patients

Parameter	African-American	Caucasian	Japanese
Number of patients studied			
Dose-Cu(S)–INR relationship	36	157	172
Plasma normal prothrombin	21	54	91
Genotyping of *CYP2C9* and *VKORC1*	(64)*	115	64
Gender (M/F)	12/24	87/70	101/71
Age (years)	61 ± 11	65 ± 13	61 ± 10†
Body weight (kg)	89.5 ± 26.4§	73.7 ± 17.1	56.5 ± 10.9†,‡
Dose of racemic warfarin (mg/day)	5.3 ± 2.6	4.7 ± 2.4	3.5 ± 1.6†,‡
Cu(S) (ng/ml)	6.76 ± 2.97§	4.09 ± 2.08	2.19 ± 1.25†,‡
CLpo,u(S) (ml/min)	314.7 ± 163.1§	469.4 ± 294.4	654.3 ± 376.8†,‡
INR	2.67 ± 0.81	2.50 ± 0.89	1.84 ± 0.59†,‡
INR/Cu(S) (ml/ng)	0.46 ± 0.21§	0.75 ± 0.45	1.05 ± 0.58†,‡
Normal prothrombin level (μg/ml)	54.6 ± 23.2	60.3 ± 36.1	52.5 ± 26.1

Abbreviations: Cu(S), plasma unbound concentration of S-warfarin; CLpo,u(S), unbound oral clearance of S-warfarin; INR, international normalized ratio of prothrombin time.
Data are mean values ± SD.
*DNA samples were obtained from healthy subjects.
†$P < 0.01$ between the Caucasian and Japanese groups.
‡$P < 0.01$ between the Japanese and African-American groups.
§$P < 0.05$ between the Caucasian and African-American groups.

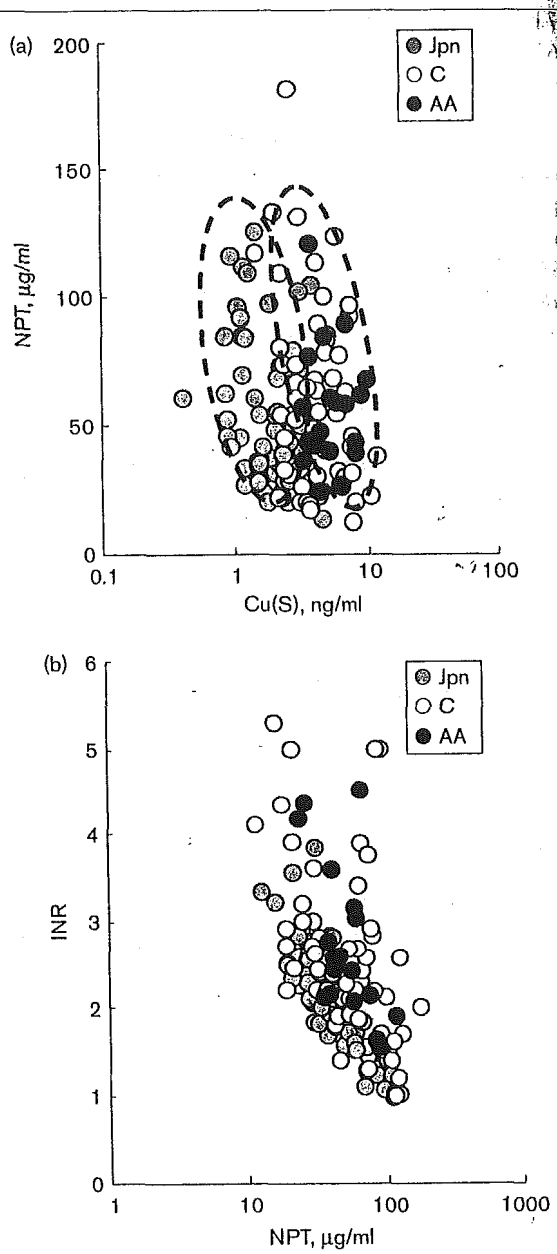
in the oral clearance of unbound S-warfarin (Table 1). No apparent differences in unbound S-warfarin's oral clearance were observed between patients who were given either amiodarone (458 ± 98 ml/min, $n = 4$) or thyroid hormone (330 ± 119 ml/min, $n = 6$) with warfarin and those were given warfarin alone. There was a significant ($P < 0.0001$) correlation between the oral clearances of S-warfarin and R-warfarin ($r = 0.706$).

Population differences were also apparent in the associated measures of anticoagulation (Table 1) with INR values in the Japanese patients being lower than in either of the other two populations. However, the 'warfarin sensitivity index' – a measure of the degree of anticoagulation normalized for the unbound S-warfarin plasma concentration – was higher in Japanese compared to Caucasians or African-Americans. No significant differences were present in the NPT concentrations between the populations; however, the distribution of NPT levels in the Japanese patients relative to the unbound plasma concentration of S-warfarin was shifted to the left compared to that in the Caucasian and African-American populations (Fig. 1a). On the other hand, the relationships between the NPT level and INR value in the three populations overlapped each other (Fig. 1b).

Seven allelic variants in the *VKORC1* gene were identified and these all exhibited differences in frequency between the populations studied (Table 2). With the exception of the 1173C > T transition in Japanese, Hardy–Weinberg equilibrium was present. A synonymous 3462C > T transition (Leu120Leu) in exon 3 was selectively present in African-Americans and two heterozygous cases of an exon 2 substitution (1331G > A, Val66Met) were also found in this population. In contrast, the transitions at 129C > T in exon 1, 497T > G in intron 1 and 1196G > A in intron 1 appeared to be present in Caucasians at a low frequency and the allelic frequencies of the transition at 3730G > A in the 3′-downstream region was significantly higher in African-American and Caucasians compared with Japanese. The most common allelic variant with a significant difference in frequency in all three populations was an 1173C > T polymorphism in intron 1 which was found in 8.6% of African-Americans, 42.2% of Caucasians and 89.1% of Japanese. Population differences in the allelic frequencies of the various *CYP2C9* variants were also found (Table 2); *CYP2C9* variants with reduced metabolizing ability were present at higher frequencies in Caucasians and African-Americans compared with Japanese.

Low but statistically significant ($P < 0.05$) correlations were present between the INR value and the unbound plasma concentrations of S-warfarin in *VKORC1* 1173 C > T heterozygotes and variant homozygotes but not homozygote wild-type in the collective results from all patients (Fig. 2). For any given genotype, the data from

Fig. 1



Relationships between plasma unbound concentrations of S-warfarin [Cu(S)] and plasma concentrations of fully carboxylated normal prothrombin (NPT) (a) and those between plasma concentrations of NPT and INR (b) in Caucasian (open circles), Japanese (grey or halftone circles) and African-American (closed circles) patients.

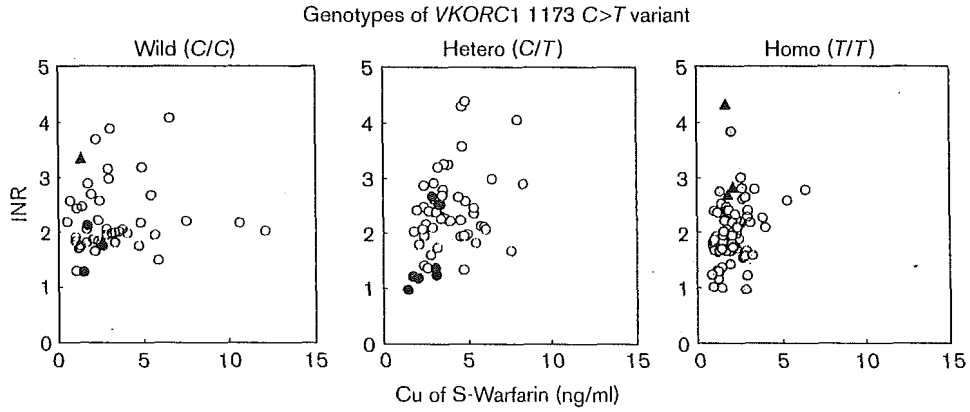
the Caucasians and Japanese patients overlapped. Additionally, the slopes of the relationships were steeper in the heterozygous and homozygous variant groups (0.163 and 0.183 ml/ng, respectively) than in the wild-type population (0.021 ml/ng). Regarding the novel *VKORC1* 1196 G > A transition, all four such Caucasian patients had an INR value greater than 2.5 at an unbound plasma concentration of S-warfarin < 5 ng/ml (i.e., they had increased warfarin sensitivity). Three of them also carried

Table 2 Allelic frequencies of *VKORC1* and *CYP2C9* variants

	African-American (n=64)	Caucasian (n=115)	Japanese (n=64)
VKORC1 129 C>T (Cys43Cys, exon 1)	0	0.009	0
VKORC 497T>G (intron 1)	0.039§	0.288	0†
VKORC1 1173C>T (intron 1)	0.086§	0.422	0.891†,‡
VKORC1 1196G>A* (intron 1)	0	0.017	0
VKORC1 1331G>A (Val66Met, exon 2)	0.016	0	0
VKORC1 3462C>T (Leu120Leu, exon 3)	0.227§	0.004	0‡
VKORC1 3730G>A 3′-downstream)	0.523§	0.374	0.167†,‡
*CYP2C9*1 (wild-type) (Arg_{144}/ Arg_{335}/Ile_{359})	0.953§	0.743	0.984†
*CYP2C9*2* (exon 3) (Arg/Cys_{144})	0§	0.143	0†
*CYP2C9*3* (exon 7) (Ile/Leu_{359})	0.008§	0.109	0.016†
*CYP2C9*4* (exon 7) (Ile/Thr_{359})	0	0	0
*CYP2C9*5* (exon 7) (Asp/Glu_{360})	0.008	0	0
*CYP2C9*6* (exon 5) (818delA)	0.008	0	0
*CYP2C9*11* (exon 7) (Arg/Trp_{335})	0.023	0.004	0

African-American DNA samples were obtained from healthy subjects.
*a novel polymorphism.
†P<0.01 between the Caucasian and Japanese groups.
‡P<0.01 between the Japanese and African-American groups.
§P<0.05 between the Caucasian and African-American groups.

Fig. 2

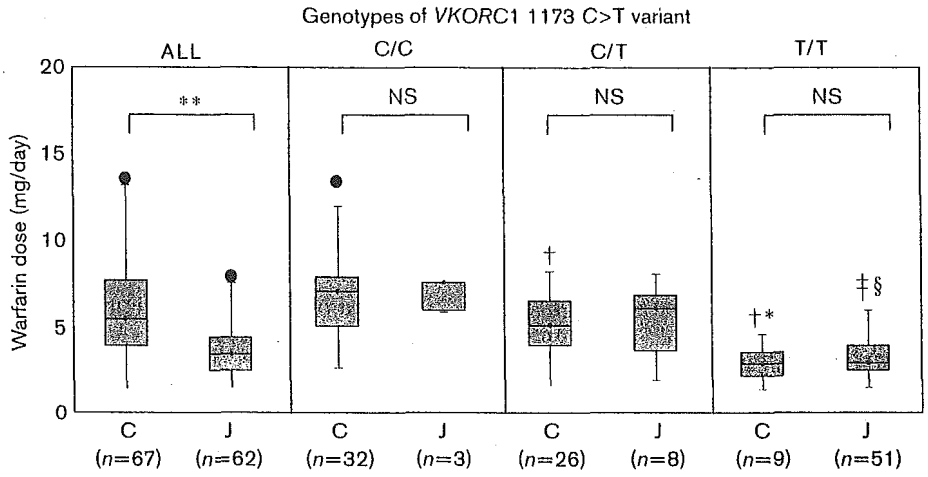


Relationships between plasma unbound concentrations (Cu) of S-warfarin and INR in Caucasian (open circles) and Japanese (grey or halftone circles) patients with three different genotypes of *VKORC1* 1173C>T: those with the wild-type (C/C), heterozygote (C/T) and homozygote (T/T) are shown separately. Four Caucasian patients carrying *VKORC1* 1196G>A are presented by black triangles. Significant (P<0.05) and apparently steeper correlations between the two parameters were observed in the C/T (r=0.35) and T/T genotypes (r=0.36), respectively.

the *VKORC1* 1173 homozygous mutant allele (T/T), but one had the 1173 wild-type genotype. No differences in metabolizing ability, as measured by the oral clearance of unbound S-warfarin, were observed between the three *VKORC1* 1173 C > T genotype groups in Caucasians and Japanese. However, reduced maintenance doses of warfarin in patients carrying *CYP2C9*2* and/or *CYP2C9*3* were observed in the Caucasians and Japanese patients (5.5 ± 2.6, 4.0 ± 1.8, 3.2 ± 1.5, 2.0 ± 1.3 mg/day in Caucasians with *CYP2C9*1/*1*, **1/*2*, **1/*3* versus **2/*3* or *versus *2/*2* or versus **3/*3*, respectively, and 3.6 ± 1.7 and 1.8 ± 0.5 mg/day in Japanese with *CYP2C9*1/*1* and **1/*3* genotypes, respectively). In order to perform further genotype: phenotype analysis (Fig. 3), patients homozygous for the wild-type *CYP2C9* gene (67 Caucasian and 62 Japanese patients) were selected to exclude the influence of population differences in the frequencies of defective *CYP2C9*2* and *CYP2C9*3* alleles on the maintenance doses.

The median daily warfarin dose in Caucasians was significantly greater (P < 0.01) than that in Japanese (5.5 versus 3.5 mg/day, respectively), when the two such populations were compared irrespective of *VKORC1* genotype (ALL in Fig. 3). There was a significant (P < 0.05) *VKORC1* 1173C > T gene–dose effect present in each population, e.g., a lower dose was observed in patients carrying homozygous mutations (T/T) compared with those with wild-type (C/C) and heterozygous mutations (C/T) except for Japanese patients with C/C

Fig. 3



Comparisons of the median maintenance doses of warfarin between Caucasian (C) and Japanese (J) patients carrying the wild-type *CYP2C9* genotype. Comparisons were made irrespective of *VKORC1* 1173C>T genotypes (ALL) and with regard to the *VKORC1* 1173C>T genotype (C/C, C/T and T/T, respectively) between Caucasian and Japanese patients. Data are shown by box-and-whisker plots. Subdivisions of the boxes and the top and bottom lines on the boxes represent median values and the upper and lower quartiles, respectively. The closed circles (●) are outlying values beyond the maximum length in terms of the interquartile range. Numbers of patients in each group are shown in the parentheses. There was a significant difference in warfarin doses between Caucasian and Japanese patients when compared irrespective of *VKORC1* genotype (ALL). There were also significant differences in warfarin doses between Caucasian patients having different *VKORC1* genotypes and between Japanese patients having 1173 C/C and T/T genotypes and between patients with 1173 C/T and T/T genotypes. $^{**}P<0.01$ between the Caucasian and Japanese groups; $^{\dagger}P<0.01$ between Caucasian patients with 1173 C/C and those with C/T or T/T genotypes; $^{*}P<0.05$ between Caucasian patients with 1173 C/T and those with T/T genotypes; $^{\ddagger}P<0.01$ between Japanese patients with 1173 C/C and those with T/T genotypes; $^{\S}P<0.01$ between Japanese patients with 1173 C/T and those with T/T genotypes.

and C/T genotypes: the mean maintenance doses obtained from Caucasian patients carrying C/C, C/T and T/T genotypes were 6.9 versus 5.2 versus 3.0 mg/day, respectively, and the corresponding values obtained from Japanese patients were 7.0 versus 5.4 versus 3.3 mg/day. In contrast, no significant differences were observed between these two populations in the daily dose within each 1173C > T genotype (Fig. 3).

Haplotype frequencies were 0.156 and 0.847 for H1, 0.256 and 0 for H2, 0.363 and 0.109 for H7/H8 and 0.200 and 0 for H9 in Caucasian and Japanese patients, respectively. Haplotype analysis revealed no significant differences in warfarin doses adjusted for age, sex, body weight and *CYP2C9* genotype and 'warfarin sensitivity index' for S-warfarin between patients in Group A, i.e., with the H1 versus H2 haplotype (3.4 versus 3.5 mg/day, and 1.0 versus 1.0 ml/ng, respectively). No significant differences were observed in the corresponding values in Group B patients with the H7/H8 haplotype and those with the H9 haplotype (5.8 versus 5.2 mg/day, and 0.66 versus 0.58 ml/ng). Haplotype groups of A/A, A/B and B/B completely corresponded to the genotype groups of *VKORC1* 1173 T/T, T /C and C/C.

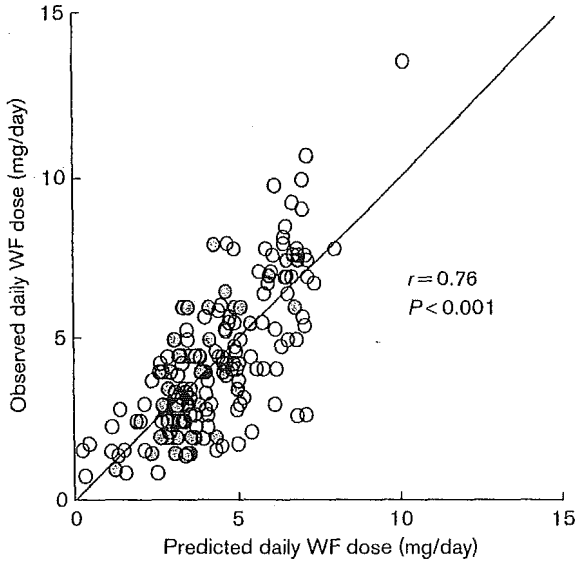
Univariate analysis to identify patient covariates associated with the interindividual variability in daily warfarin dose showed that age ($r = -0.22$), body weight ($r = 0.29$), *CYP2C9* variant ($r = -0.32$), *VKORC1* 1173C > T ($r = -0.58$) and Japanese ancestry ($r = -0.20$) were all significantly ($r < 0.05$) correlated. Further multivariate analysis with these covariates in 115 Caucasian and 64 Japanese patients revealed that *CYP2C9* and *VKORC1* genotypes, age and body weight had independent and statistically significant contributions to the overall variability in warfarin dose (Table 3). The final regression equation for estimating maintenance doses (MD) of warfarin was as follows: for patients with homozygous wild-type genotype for both *CYP2C9* and *VKORC1*: MD (mg) = 6.6 − 0.035 × (age, years) + 0.031 × (body weight, kg); for those with either heterozygous or homozygous variant of *CYP2C9*, the MD was reduced by 1.7 and 2.8 mg, respectively, and for those with either heterozygous or homozygous variant of *VKORC1* 1173C > T, the MD was further reduced by 1.3 and 2.9 mg, respectively, from those predicted by the respective equations. Based on the standardized partial regression coefficients, genotypes of *CYP2C9* and *VKORC1* were the principal covariates contributing equally to interpatient variability in warfarin requirements. Collectively, the identified covariates accounted for 57% of the overall variability in the daily dose of warfarin. Also, a significant correlation ($r = 0.76$, $P < 0.001$) without systematic bias was observed between the actual maintenance doses taken by the Caucasian and Japanese patients and those predicted from the multiple regression model (Fig. 4).

Table 3 **Multivariate analysis for patients' covariates that are associated with interindividual variability of warfarin doses**

Covariates	Partial regression coefficient ± SE	Standardized partial regression coefficient	*P*-value
Constant	6.656 ± 0.973		
Age (years)	−0.035 ± 0.010	−0.252	0.000808
Body weight (kg)	0.031 ± 0.007	0.298	0.000059
*CYP2C9*2/*3/*11* (Heterozygous)	−1.706 ± 0.290	−0.408	<0.0000005
(Homozygous variant)	−2.815 ± 0.473	−0.413	<0.0000005
VKORC1 1173 C>T (Heterozygous)	−1.316 ± 0.309	−0.310	0.000034
(Homozygous variant)	−2.941 ± 0.310	−0.590	<0.0000005

SE, standard error of mean.

Fig. 4



Relationship between maintenance doses of warfarin predicted from the multiple regression model and those actually observed in the 115 Caucasian (○) and 64 Japanese (●) patients. There is a significant correlation between the predicted and observed doses ($y=x+0.0008$, $r=0.76$, $P<0.001$). The solid line represents the line of identity.

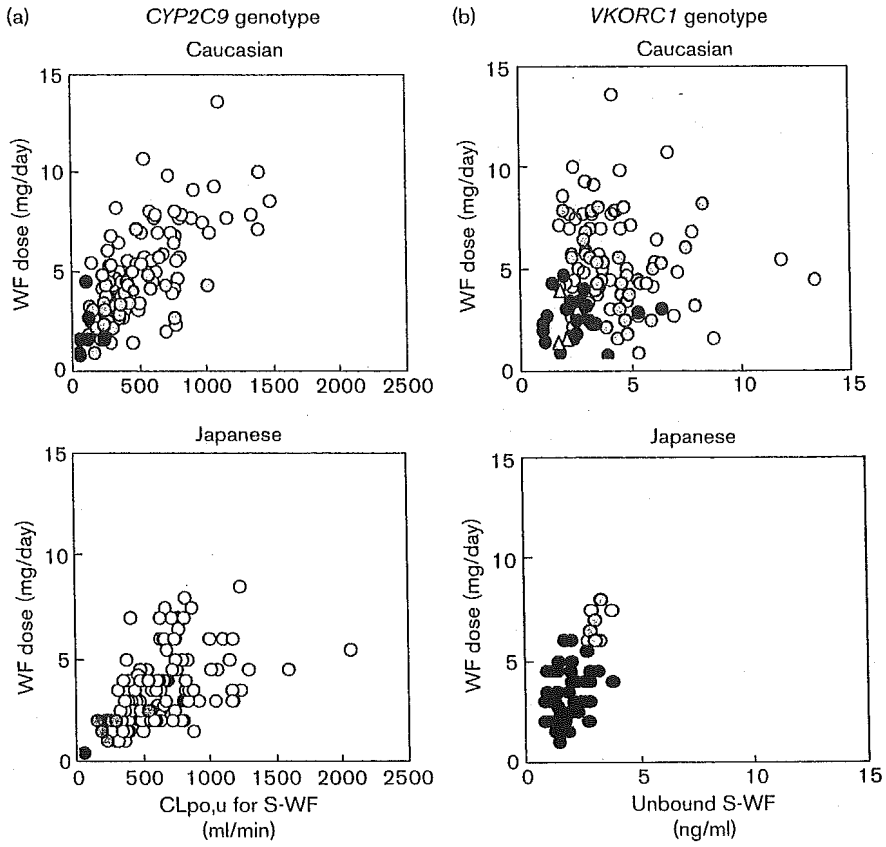
Caucasian and Japanese patients who carried *CYP2C9* variants possessed a lower unbound oral clearance for S-warfarin (decreased metabolic activity), thereby required a smaller daily dose of the drug (Fig. 5a). In addition, those carrying the *VKORC1* 1173C/C wild-type allele needed higher unbound concentrations of S-warfarin to achieve a therapeutic anticoagulation response (reduced sensitivity), and a greater daily dose was required regardless of race (Fig. 5b). Forty-seven percent of Caucasian patients possessed one of the *CYP2C9* variant alleles (*CYP2C9*2*, *CYP2C9*3* or *CYP2C9*11*) and 48% the *VKORC1* 1173 C/C wild-type allele, respectively. The corresponding values for African-Americans were 11% and 83%, and those for Japanese were 3% and 17%, respectively. These genetic polymorphisms in *CYP2C9* and *VKORC1* were independent to each other and allelic frequencies of these genetic variants differed among the three populations (Table 2). As a result, 70% of Caucasian, 83% of African-American and 20% of Japanese patients were found to carry pharmacokinetic (*CYP2C9*) and pharmacodynamic (*VKORC1*) genetic factors which are associated with a lower and a higher requirement, respectively, resulting in the wide interindividual variation in warfarin doses.

Discussion

Warfarin therapy is complicated by large interpatient variability in maintenance dose requirement and the associated risk of under- and over-anticoagulation. This is the first study demonstrating that there are population differences not only in pharmacokinetics but also in pharmacodynamics of warfarin based upon the dose-plasma concentration and plasma concentration–INR relationships. The pharmacodynamics of S-warfarin evaluated by its 'warfarin sensitivity index' showed significant differences between African-Americans, Caucasians and Japanese patients, although the number of African-American patients ($n=36$) participating in the study was smaller than the Caucasians and Japanese groups (Table 1). In addition, the sensitivity of S-warfarin to inhibit normal or fully carboxylated prothrombin (NPT) production was found to differ between populations and this may play a pivotal role in the population differences of warfarin dose requirement.

Readily determinable demographic factors such as age and body weight have been considered as contributing covariates [1–3], and this is confirmed in the present study. The age factor may be related to a reduced ability to metabolize warfarin with aging [1]. A similar mechanistic explanation may also account for the body weight covariate although a pharmacodynamic factor may also be involved, since obese subjects have been found to have elevated plasma levels of fibrinogen and factor VII compared to lean individuals [28]. Nonetheless, such demographic factors only have limited utility for optimizing the warfarin maintenance dose and it has become increasingly appreciated that genetic factors may have an important role. Recent focus has been upon drug metabolizing enzymes involved in warfarin's metabolism that influence its plasma concentration.

Fig. 5



Relationships between unbound oral clearance (CLpo,u) for S-warfarin and daily doses of warfarin (left column, a) and those between plasma unbound concentration (Cu) for S-warfarin and daily doses of warfarin (right column, b) in Caucasian and Japanese patients with different genotypes of *CYP2C9* (a) and *VKORC1* (b). Symbols (a): *CYP2C9*1/*1* (open circles), *CYP2C9*1/*2* or **1/*3* or **1/*11* (grey circles) and *CYP2C9*2/*2*, or **3/*3* or **2/*3* (black circles); symbols in (b): *VKORC1* 1173 C/C and 1196 G/G (open circles), *VKORC1* 1173 C/C and 1196 G/A (open triangle), *VKORC1* 1173 C/T and 1196 G/G (grey circles), *VKORC1* 1173 T/T and 1196 G/G (black circles) and *VKORC1* 1173T/T and 1196 G/A (grey triangles).

Clinically available warfarin is a racemic mixture of R- and S-enantiomers. However, S-warfarin has been shown to be three to five times more potent than R-warfarin based upon the anticoagulation responses elicited after the administration of the respective enantiomers separately in healthy subjects [11]. While plasma concentrations of R-warfarin are, on average, approximately twice those of S-warfarin following oral administration of the racemate, pharmacokinetic-pharmacodynamic analysis concluded that the anticoagulant effect is attributable almost entirely to S-warfarin concentrations [29]. Moreover, as noted in the present study, there was a significant correlation between the oral clearance of unbound S-warfarin and that for R-warfarin ($P < 0.0001$), indicating that demographic factors (e.g., body weight and age), nutritional and certain environmental factors linked with variability in both of these parameters may also be associated. Accordingly, it is likely that interindividual variability in the plasma concentration of S-warfarin is more important than that of R-warfarin when considering the variability of anticoagulant activity following the administration of racemic warfarin.

CYP2C9 and its allelic variants have been investigated since the encoded enzyme is largely responsible for the metabolism of S-warfarin. Several relatively large retrospective clinical studies in several different populations have now demonstrated associations between warfarin's maintenance dose and adverse events, i.e., increased bleeding complications, and the presence of *CYP2C9* variants leading to markedly reduced catalytic activity of the resulting enzyme such as CYP2C9.2 and CYP2C9.3 [1–3,9,12–14]. Collectively, the present data confirm these previous observations that lower doses are required in patients carrying these variant alleles especially *CYP2C9*3*. Despite such associations, however, the contribution of such genetic variability to the overall variability in warfarin's maintenance dose is relatively low – less than 20% of the variance [1–3]. The present findings based on the presence of *CYP2C9*2*, *CYP2C9*3*

and *CYP2C9*11* variants, all of which are associated with reduced enzyme activity, also confirm this small contribution even when variant homozygosity is present. Moreover, the difference in warfarin dosage requirement between Japanese and Caucasians cannot be explained by a greater frequency of *CYP2C9* variants with reduced catalytic activity in Caucasians (Table 2), and the former population have higher unbound oral clearances of S-warfarin than the latter when matched for the wild-type genotype in the 5′-flanking (up to −2 kb) and coding regions of *CYP2C9* [9,27]. Therefore, the present results strongly suggest the involvement of other factors.

The molecular target of warfarin is vitamin K epoxide reductase, which is critically involved in the production of functionally active vitamin K-dependent coagulation factors [e.g., factors II (prothrombin), VII, IX and X)] through γ-glutamyl carboxylation [30]. Subunit 1 of this lipoprotein complex has recently been shown to exhibit genetic polymorphisms, and several such allelic variants have been shown to have reduced catalytic activity that is associated with 'warfarin-resistance', i.e., require substantially higher doses to achieve satisfactory anticoagulation [15,17]. However, only two such heterozygous *VKORC1* 1331G > A, Val66Met, African-American individuals were found in the present study. Other variants reported to be associated with 'warfarin-resistance' [15] were not detected. A number of other nucleotide transitions including a novel *VKORC1* 1196G > A were, however, identified and appeared to have selective distribution according to racial ancestry, but their rarity made it impossible to assess whether they have functional consequences. On the other hand, a haplotype combination including a *VKORC1* 1173C > T transition, previously reported to be present in 40% of European-Caucasians, was found to be common with higher and lower frequencies in Japanese and African-Americans, respectively [16–21]. This variant was also found to be associated with a gene–dose effect and a lower warfarin maintenance dose [16–21]. The present findings confirm this observation in Caucasians and extend the relationship to Japanese. Interestingly, this *VKORC1* variant appeared to affect the relationship between the unbound concentrations of S-warfarin and the resulting INR value – the slopes of the regression curves of the relationship being steeper in heterozygous and homozygous variant patients than in those homozygous for the wild-type allele. Importantly, the different population frequency of the *VKORC1* 1173T variant allele in Japanese compared to Caucasians, appeared to account for the increased 'warfarin sensitivity' of the former group of patients, matched according to *CYP2C9* genotype, i.e., *CYP2C9*1* homozygous, since no differences in dosage requirement was observed between the populations when stratified according to *VKORC1* genotype. Furthermore, multiple regression analysis showed that the *VKORC1* 1173C > T variant was an important covariate with respect to the interindividual variability in warfarin dosage. Patients carrying the T allele at the position of 1173 of *VKORC1* gene are classified into the Group A haplotype associated with a lower dose requirement [21]. However, this haplotype system is no more informative than a single segregating SNPs among those at positions 381, 3673, 6484, 6853 and 7566 of the reference sequence (GenBank accession number AY587020) as shown previously by others [16], when the influence of *VKORC1* genotype on the interindividual variability in warfarin doses is considered. Overall, these results also suggest that the higher dose requirements in African-Americans [6,7] may possibly reflect the higher frequency of the *VKORC1* 1173C allele (91%) compared to Japanese (11%) and Caucasians (58%) (Table 2).

The 1173C > T transition in intron 1 of *VKORC1* was recently reported to be in complete disequilibrium with −1639G > A at a putative NF1 binding site [18], −4931T > C, 1542G > C and 2255C > T [21]. While there is a controversy regarding the influence of this *VKORC1* haplotype on the transcriptional activity of this gene [16,18,19], a recent report indicates that this haplotype was associated with lower mRNA levels in human liver [21]. This finding suggests that the 1173C > T variant may be associated with the lower levels of reduced form of vitamin K, thereby making patients with this variant more susceptible to the anticoagulation effect of warfarin. In addition to the conventional measure of anticoagulation, namely, the INR value, the concentration of NPT was also determined in the patients. No population differences could be discerned in the relationship between these two biomarkers, indicating comparable functionality of the involved fully carboxylated vitamin K-dependent factors and fibrinogen. However, Japanese patients appeared to be more sensitive to γ-carboxylation of prothrombin in that a comparable NPT response was achievable at lower plasma concentrations of unbound S-warfarin compared to Caucasians and African-Americans. The reason for this difference is unknown but may involve population differences in NPT's baseline level (preliminary unreported data), and further studies are required to explore this possibility. In addition, the question of whether the *VKORC1* haplotypes may influence the baseline levels of VKOR and NPT remains to be clarified. Regarding functionally related genes, multiple variants in several vitamin K-dependent proteins have been identified including factor II, factor VII and γ-glutamyl carboxylase [20,31]. Moreover, some of these are associated with altered 'warfarin sensitivity' [20,31] and preliminary data (not shown) indicates that their allelic frequencies differ between Caucasian and Japanese populations. Therefore, influences of these polymorphisms on the overall variability in warfarin responses are also to be clarified.

In summary, the present study shows that interindividual variability and population differences in the maintenance dose of warfarin required to achieve anticoagulation involves demographic, pharmacokinetic, and pharmacodynamic factors. Furthermore, genetic variability in CYP2C9-mediated metabolism of S-warfarin and the drug's molecular target, VKOR, are specific determinants. The present study shows that 70% Caucasian and 83% African-American patients carried either *CYP2C9* or/and *VKORC1* genotype(s) which leads to either reduced metabolic activity or attenuated sensitivity of warfarin. In contrast, only 20% of Japanese population possesses these genotypes. Thus, the relative contribution of the *VKORC1* and *CYP2C9* genotypes to the overall interpatient variability in warfarin doses differs between the three populations according to racial ancestry. Moreover, it should be of note that the identified demographic and genetic covariates of warfarin doses only account for 57% of interindividual variability. Accordingly, other currently unknown determinants remain to be identified, and populations other than those currently studied need to be investigated.

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Population Pharmacokinetic and Pharmacodynamic Analysis of a Class IC Antiarrhythmic, Pilsicainide, in Patients With Cardiac Arrhythmias

Ryuichi Ogawa, MSc, Ryoji Kishi, MD, Kiyoshi Mihara, PhD, Harumi Takahashi, PhD, Akihiko Takagi, MD, Naoki Matsumoto, MD, Keisou Masuhara, PhD, Kiyoshi Nakazawa, MD, Fumihiko Miyake, MD, Shinichi Kobayashi, MD, and Hirotoshi Echizen, MD, PhD

Population pharmacokinetics (PK) of a sodium channel–blocking antiarrhythmic, pilsicainide, was studied using the nonlinear mixed-effects modeling technique in 91 patients with cardiac arrhythmias (80 suspected Brugada syndrome [BrS] and 11 with atrial fibrillation) who received an intravenous infusion of 10 mg of the drug. Population pharmacodynamic (PD) analysis was also performed using an effect compartment model. PD responses were assessed by changes in electrocardiogram (ECG) pattern (BrS-like elevation of ST-segment) and conduction parameters. The final PK model showed that gender (values were 50% lower in women than in men) and creatinine clearance were significant (P < .01) covariates of weight-normalized systemic clearance of pilsicainide. Patients who showed a BrS-like ECG pattern after the drug administration also showed a significantly (P < .01) greater prolongation in His-Purkinje conduction compared to the remaining patients. In conclusion, female gender, renal dysfunction, and the drug-induced BrS-like ECG morphology may be associated with augmented ECG responses to pilsicainide.

Keywords: *Pilsicainide; pharmacokinetics-pharmacodynamics; Brugada syndrome*

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Pilsicainide HCL is a class IC antiarrhythmic drug widely used in the treatment of supraventricular and ventricular tachyarrhythmias in Japan. Previous in vitro and in vivo studies have revealed its unique pharmacokinetic (PK) and pharmacodynamic (PD) characteristics. Electrophysiological studies[1,2] performed with isolated myocardial cells using standard microelectrode and whole-cell clamp techniques revealed that pilsicainide is a pure sodium channel blocker with no autonomic blocking effects and no potassium/calcium channel–blocking properties. The conventional PK study[3] of the drug performed during a phase 1 clinical trial in healthy young male subjects demonstrated that it is eliminated mainly into urine in unchanged form and that an active tubular transport is likely to be involved. The renal clearance (200-300 mL/min) of pilsicainide surpasses the glomerular filtration rate (100 mL/min). Since the drug is a cationic drug, an active tubular secretion mediated by one of the organic cation transporters (OCTs) may be associated with its renal elimination. To our knowledge, however, few efforts have been made to identify clinical covariate(s) dominating interindividual variability of PK and/or PD of this antiarrhythmic agent.

The necessity of a PK/PD study of pilsicainide has been fueled by recent clinical findings indicating that this drug may serve as a useful probe for assessing altered sodium channel responsiveness in patients sus-

From the Departments of Pharmacotherapy (Mr Ogawa, Dr Takahashi, Dr Echizen) and Biopharmaceutics (Dr Mihara), Meiji Pharmaceutical University, Tokyo; and the Departments of Cardiology (Dr Kishi, Dr Takagi, Dr Nakazawa, Dr Miyake), Pharmacology (Dr Matsumoto, Dr Kobayashi), and Hospital Pharmacy (Dr Masuhara), St. Marianna University School of Medicine, Kawasaki, Japan. Submitted for publication May 18, 2005; revised version accepted September 30, 2005. Address for reprints: Hirotoshi Echizen, MD, PhD, Department of Pharmacotherapy, Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose, Tokyo 204-8588, Japan.
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pected of Brugada syndrome (BrS).[4] BrS is considered to be a sodium channelopathy associated with a high incidence of sudden death due to fatal arrhythmias developing in subjects with structurally normal hearts. The condition is more prevalent in Asians than in whites. Characteristic electrocardiogram (ECG) patterns consisting of right bundle branch block and either coved or saddleback-shape ST-segment elevation in the right precordial leads (ie, V_1-V_3) are hallmarks of BrS. Since the ECG findings obtained from patients with BrS show variations over time in the same patient (eg, either exaggeration or amelioration by autonomic interventions),[5] it is difficult to arrive at a conclusive diagnosis of BrS in patients with apparently normal or equivocal ECG findings despite highly suspicious clinical manifestations and presence of family history of the disease. For such patients, a pharmacological provocation test with a pure sodium channel blocker such as pilsicainide may be of value. Nevertheless, there is a paucity of knowledge about PK/PD covariates that contribute to the interindividual variability in responsiveness to the drug. In this context, we decided to undertake a population PK/PD analysis of pilsicainide in Japanese patients with cardiac arrhythmias using nonlinear mixed-effects modeling (NONMEM).[6] In this article, we present data indicating that gender and renal function are the major determinants of the PK variability of pilsicainide and that drug-induced BrS-like ST-segment elevation in ECGs may be a phenotypic trait of exaggerated dromotropic effects in response to the drug.

METHODS

Patients and Study Design

Ninety-one patients received an intravenous administration of pilsicainide in the coronary care unit of St. Marianna University School of Medicine Hospital in Kawasaki, Japan. Eighty patients received the drug as a diagnostic test for BrS, and 11 patients received the drug for controlling paroxysmal atrial fibrillation. The study protocol had been approved by the Institutional Review Board of St. Marianna University School of Medicine before the study was started. Written informed consent was obtained from each patient after the purpose of the study and possible risks and benefits were thoroughly explained.

Each patient was given an intravenous infusion of pilsicainide HCL at a rate of 1 mg/kg over 10 minutes under continuous ECG monitoring.[4] The patients who were given the drug for diagnosing BrS were further divided into 2 groups according to the ECG responses to the drug. Taking the criteria proposed by Brugada et al[7] into consideration, we tentatively assigned those developing drug-induced ST-segment elevation of +0.15 mV or greater from the baseline ECG tracing at J point (at the end of QRS complex), ST_{80} point (at 80 milliseconds after the end of QRS complex), or QT_{160} point (at 160 milliseconds after the beginning of QRS complex) in the V_2 lead of the standard 12 leads ECG as responders to pilsicainide (group A). The remaining patients were considered nonresponders (group B). Plasma pilsicainide concentrations obtained from the patients who received the drug for treatment of atrial fibrillation were used exclusively for the PK analysis. Blood biochemistry and urinalysis were performed at the Department of Clinical Chemistry, St. Marianna University School of Medicine Hospital.

Blood Samplings and ECG Recordings

Most blood samples (5 mL each) were obtained within 120 minutes after the end of pilsicainide infusion under continuous ECG monitoring. At least 2 samples were obtained from all but 6 of the patients during this period. Additional blood samples were obtained thereafter up to 24 hours postdose when possible. Blood was collected into glass tubes containing EDTA-2Na, and plasma was separated immediately by centrifugation at 1630*g* for 10 minutes at 4°C and stored at –20°C until analyzed.

Continuous ECG monitoring was performed during the study, and ECGs were recorded at a paper speed of 25 mm/s at 5 minutes before the pilsicainide infusion was started (baseline) and at 0, 5, 10, 30, 60, 90, and 120 minutes after completion of drug infusion. The pharmacological effects of pilsicainide on electrical conduction in the heart were assessed by changes in P wave duration, PQ interval, PEQ interval, and QRS duration. PEQ interval is defined as the isoelectrical region from the end of the P wave to the onset of the QRS complex. It largely represents the period associated with impulse propagation from the AV node to the His-bundle and intraventricular conduction system. Measurements of these parameters were made by one of the authors (R.O.) using a digital vernier caliper (Mitsutoyo Co, Tokyo, Japan) for at least 5 consecutive beats at each sampling point, and the mean value was calculated. Both within- and between-day intraobserver variability of measurements assessed as coefficients of variation (CVs) were <2%. The respective ECG parameters at each sampling time were expressed as degrees of change from the corresponding baseline values.

Pilsicainide Assay

Plasma pilsicainide assay was performed with a high-performance liquid chromatography–ultraviolet absorption according to Shiga et al[8] with minor modifications. Briefly, we used quinidine (final concentration of 1.0 μg/mL) as internal standard and a reversed-phase column (Capcel-Pak C_{18}, 5 μm, 250 × 4.6 mm; Shiseido Co Ltd, Tokyo, Japan) for the analysis. The mean (±SD) percent recovery of pilsicainide and the internal standard from extraction were 101% ± 4% and 105% ± 4%, respectively. Calibration curves were linear over the drug concentration range of 0.05 to 1.0 μg/mL ($r > 0.999$, $P < .01$). The within- and between-day precisions for the assay were <5% as the CV and the accuracy ranged from −9% to +16% as percentage error from the theoretical concentrations ranging from 0.05 to 1.0 μg/mL.

Population PK Analysis

The population PK analysis was performed on 237 plasma concentrations of pilsicainide obtained from 91 patients by applying the NONMEM (version V, level 1.0; University of California, San Francisco).[6] A preliminary study using not only the objective function (OBJ) values but also the distribution of weighted residues to evaluate the goodness of fit of PK models indicated that the 2-compartment model with zero-order input and first-order elimination from the central compartment had a better fit than the 1-compartment model did. Therefore, further analysis was performed by the 2-compartment model. The linear 2-compartment structural model was parameterized in terms of the primary PK parameters, comprising systemic clearance (CL), volumes of the central and peripheral compartments (V_c and V_p, respectively), and intercompartmental clearance (Q) using a part of the NONMEM program (PREDPP subroutines ADVAN3 and TRANS4, the first-order conditional estimate method). Compilation of the program was performed with DIGITAL Visual Fortran (Professional Edition, version 6.0A; Digital Equipment Corp, Nashua, NH). A preliminary analysis performed with a basic model showed that CL, V_c, and V_p, but not Q, were dependent on body weight. Therefore, body-weight-normalized parameters were used for CL, V_c, and V_p in the subsequent analyses. The reason Q was independent of body weight remains unclear. The choice of statistical models for the interpatient and residual (intrapatient) variability were made based on the OBJ values and the distribution of the weighted residuals as a function of patients' individual post hoc estimates of plasma pilsicainide concentrations obtained from the different error structures (ie, proportional, exponential, or additive). Since the results indicated that the proportional error model fitted to the data better than the other models did, we adopted the proportional error model for the analysis of the interindividual and residual variances in the PK of pilsicainide.

Then, we assessed whether incorporation of patients' parameters (age, gender, serum creatinine, and predicted creatinine clearance) as covariates of CL and V_p would reduce the interindividual variability assessed by the OBJ value. Particular caution was exercised to select covariates that were mutually independent. For instance, the Cockcroft-Gault equation[9] used for estimating creatinine clearance depends on age and serum creatinine concentration. Thus, creatinine clearance, rather than age and serum creatinine, was selected as a possible covariate for CL of pilsicainide. In addition, because the distribution of pilsicainide occurs rapidly (typically within 5 minutes after the end of infusion) and only a limited number of data points were available during this period, covariate analysis was not done for V_c. Regarding the model selection for continuous covariates, linear ($P = \theta_1 + \theta_2 \cdot Fac$), reciprocal ($P = \theta_1 + \theta_2/Fac$), power ($P = \theta_1 + Fac^{\theta 2}$), and maximum effect ($P = \theta_1 + \theta_2 \cdot Fac/[\theta_3 + Fac]$) equations were tested, where P represents PK parameters (such as CL), Fac represents the measurements of relevant covariates, and θ_x are the estimates calculated by NONMEM. For a categorical covariate (such as gender), the equation $P = \theta_1 \cdot (1 - Fac) + \theta_1 \cdot \theta_2 \cdot Fac$ was used, where Fac equals 0 for men and 1 for women. During model building, a reduction in the OBJ value of at least 6.635 ($\alpha = .01$) after incorporating a single covariate was considered statistically significant. Model building was performed by a stepwise extension of the model, adding an additional covariate at each step. The validity of a full model was checked by a stepwise backward elimination of each parameter. The goodness of fit of the final population PK model was also assessed by inspecting the scatter plots of population model-predicted as well as the observed pilsicainide concentrations and weighted residual as a function of population model-predicted pilsicainide concentrations. The accuracy and robustness of the final population PK model were assessed by use of a bootstrap method.[10] From the original data set of 91 patients, 400 bootstrap sets of 91 individuals were drawn by resampling. For each of the 400 bootstrap sets, the population PK parameters were estimated and then compared with those obtained in the original data set. The

final model was considered validated if no significant differences were observed.

Sequential Population PK/PD Analysis With Effect Compartment

Because no measurements of intraatrial conduction time (eg, P wave, PQ and PEQ intervals) were possible in patients with atrial fibrillation (group C), population PD analysis was conducted only in patients of groups A and B. PD responses in terms of ECG parameters elicited after pilsicainide infusion were plotted against the plasma drug concentrations obtained from actual measurements or individual post hoc estimates generated by applying the NONMEM. Since visual inspection showed that PD responses lag behind plasma drug concentrations (ie, counterclockwise hysteresis), the drug concentration-effect relationship was analyzed by the so-called effect compartment model developed by Sheiner et al.[11] According to this model, K_{e0}, defined as the elimination rate constant of drug in the effect compartment, characterizes the time-dependent aspects of equilibrium between plasma concentration and effect. The sigmoid E_{max} model, in which the concentration is substituted by the effect site concentration (Ce), was fitted to the time course of PD responses by the NONMEM program. The choice of the statistical model for error structure and the analysis of patient characteristics (age, gender, the presence or absence of ST-segment elevation) relevant to the PD variability were performed as outlined in the PK analysis. The model-building process and the criteria for selecting an optimal model are essentially similar to those for PK analysis as described above.

Statistical Analysis

Multiple comparisons in the demographic and baseline ECG parameters among the 3 groups were made by ANOVA followed by the 2-sided unpaired *t* test with Bonferroni's correction. For comparisons of proportions, either a χ^2 test or Fisher exact test was used where appropriate. The least-squares regression method was used for assessing a correlation between creatinine clearance and systemic clearance of pilsicainide, those between measured plasma drug concentrations and PD responses and those predicted by the NONMEM method. Statistical analyses were performed by the SPSS 7.5J program (SPSS Inc, Chicago, Ill). A *P* value of less than .05 was considered statistically significant. Data are expressed as means ± SD (range) throughout the study.

RESULTS

Patients

Table I lists the demographic and clinical characteristics (eg, baseline ECG parameters and complications) of the patients who participated in the present study. The mean age of group C was significantly ($P < .05$) greater than that of group A. In addition, the mean predicted creatinine clearance in group C was significantly ($P < .05$) smaller than that in group A. In agreement with previous reports,[12] men were predominant over women in patients exhibiting ECG findings compatible with or suspected of BrS (group A).

Population PK Analysis

Figure 1a and its inset show scatter plots of plasma pilsicainide concentrations versus time. Gender and CL_{cr} were found to be significant ($P < .01$) covariates for CL of pilsicainide in the final population PK model, as was age for V_p. Table II lists the respective population PK parameters, coefficients of covariates possessing significant fixed (ie, systematic) effects on the PK parameters, and random effect parameters (ie, inter- and intraindividual variance and their coefficient of variations). The final population PK model for CL and V_p is represented by the following equations:

$$CL_{TV} = (\theta_1 + \theta_2 \cdot CCR) \cdot (1 - SEX) + (\theta_1 + \theta_2 \cdot CCR) \cdot \theta_3 \cdot SEX, \quad (1)$$

$$V_{pTV} = \theta_4 + \theta_5 \cdot AGE, \quad (2)$$

where CL_{TV} is the typical value (ie, population mean) of body-weight-normalized CL of pilsicainide (L/min/kg), CCR is the predicted CL_{cr} (L/min/kg), SEX is the gender parameter (ie, 0 = male, 1 = female), θ_1 and θ_2 are the intercepts as a function of total body weight and slope parameters for the relationship between CL_{cr} and CL for male patients, θ_3 is the coefficient of CL for women, V_{pTV} is the typical value of peripheral volume of distribution in liters per kilogram, AGE is the age of patients in years, and θ_4 and θ_5 are the intercepts as a function of total body weight and slope parameters for the relationship between age and V_p. Taking the significant patients' covariate into account, the interindividual variability of CL, V_c, Q, and V_p and the residual variability assessed as CVs were 14.1%, 31.8%, 41.8%, and 25.2%, respectively. There was a good agreement between plasma pilsicainide concen-

Table I Demographic and Clinical Characteristics of Patients Who Underwent the Pilsicainide Challenge Test for the Diagnosis of Brugada Syndrome (Groups A and B) and Those Who Received the Drug for the Treatment of Paroxysmal Atrial Fibrillation (Group C)

Patient Characteristic	Patients With Suspicious Brugada Syndrome		Patients With Paroxysmal Atrial Fibrillation (Group C)
	Group A (Responders)	Group B (Nonresponders)	
Elevation of ST-segment[a]	Present	Absent	Not applicable
Number of subjects	36	44	11
Number of plasma samples	104	109	24
Gender, M/F	32/4	35/9	7/4
Age, y	48 ± 17 (19-79)	56 ± 14 (25-78)	66 ± 8* (57-76)
Height, cm	166 ± 8 (150-180)	165 ± 8 (148-179)	163 ± 7 (148-175)
Body weight, kg	62 ± 10 (39-84)	62 ± 11 (43-85)	66 ± 9 (54-77)
Body mass index, kg/m^2	22 ± 3 (17-32)	23 ± 3 (15-30)	25 ± 3 (21-29)
Renal function			
Serum creatinine, mg/dL	0.80 ± 0.17 (0.52-1.10)	0.81 ± 0.22 (0.42-1.28)	0.87 ± 0.19 (0.60-1.20)
Blood urea nitrogen, mg/dL	15 ± 4 (9-26)	15 ± 4 (8-24)	17 ± 2 (12-19)
Predicted creatinine clearance, mL/min	96 ± 31 (49-168)	88 ± 28 (39-150)	68 ± 15* (45-92)
Baseline ECG parameter			
P wave, ms	105 ± 16 (72-137)	107 ± 17 (68-142)	NA
PQ interval, ms	179 ± 31 (122-265)	174 ± 23 (133-236)	NA
PEQ interval, ms	75 ± 24 (31-150)	67 ± 22 (32-135)	NA
QRS complex, ms	90 ± 16 (64-128)	94 ± 20 (60-148)	NA
J point, mV	0.24 ± 0.25 (–0.10-0.96)	0.19 ± 0.28 (–0.16-1.89)	NA
ST_{80} point, mV	0.25 ± 0.16 (–0.15-0.62)	0.23 ± 0.13 (–0.13-0.54)	NA
QT_{160} point, mV	0.23 ± 0.15 (–0.15-0.54)	0.21 ± 0.10 (–0.08-0.47)	NA
Complication, no. of patients (%)			
Bradycardia	2 (6)	6 (14)	2 (18)
Ischemic heart disease	0 (0)	4 (9)	3 (27)
Cardiomyopathy	2 (6)	5 (11)	1 (9)
Diabetes mellitus	2 (6)	3 (7)	1 (9)
Thyroid disorder	1 (3)	2 (5)	2 (18)
Seizure	3 (8)	0 (0)	0 (0)

a. When ST-segment elevations of 0.15 mV or greater from the baseline of ECG tracing were observed either at the J, ST_{80}, or QT_{160} point in the V2 lead of the standard 12 leads, the response was considered positive. Data are presented as means ± SD (range).
*$P < .05$ versus group A.

trations predicted by the final population PK model and the observed concentrations (Figure 1b). In addition, when weighted residuals for pilsicainide concentrations predicted by the final population PK model were plotted as a function of its log-transformed plasma concentrations, the data appear to distribute uniformly around the line of Y = 0 (Figure 1c), indicating that there is little concentration-dependent bias in the estimation of the plasma drug concentration. The model validation performed with bootstrapping showed that the mean parameter estimates were within –18% and +26% of those obtained with the original data set. In addition, the 95% confidence intervals of the PK parameters obtained with bootstrapping spanned the corresponding parameters obtained in the original data set.

Sequential Population PK/PD Analysis With Effect Compartment Model

Three patients (1 in group A and 2 in group B) were excluded from the PD analysis because they developed atrial fibrillation after the infusion of the drug. Table III summarizes the number of PD data points; the error models used to describe the interindividual variance; the improvements of the OBJ value from the basic model; the population mean of K_{e0} estimated by the final effect compartment PK/PD model, E_{max}, EC_{50}, and

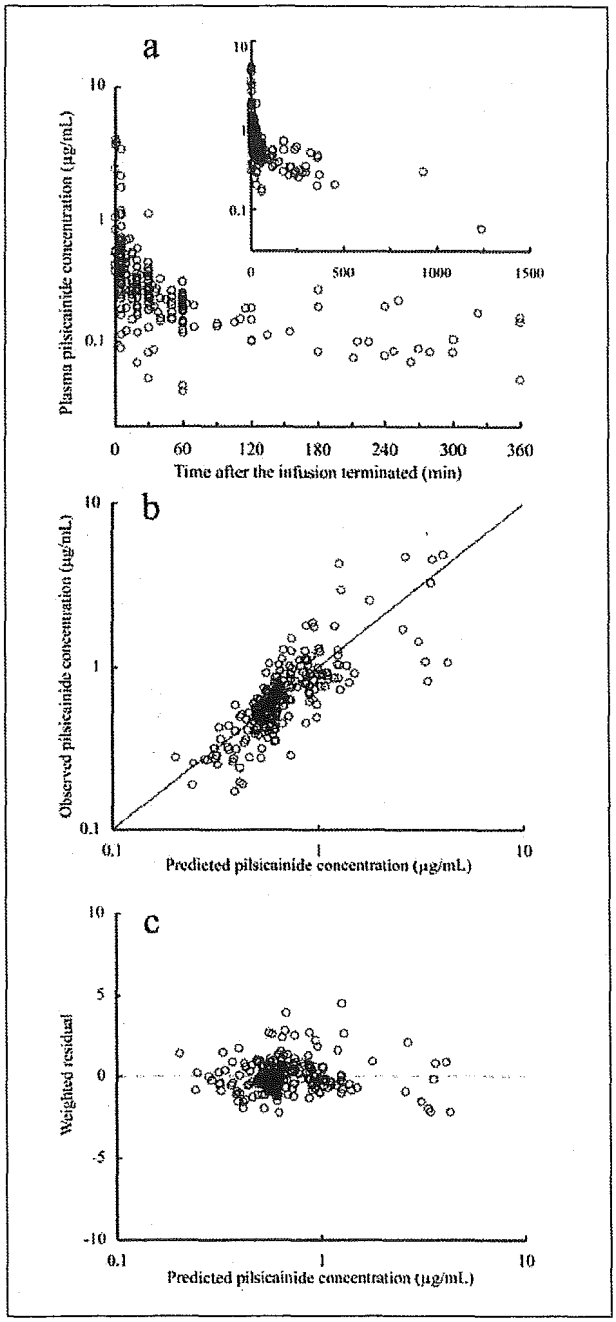

Figure 1. (a) The plasma concentration-time plots of pilsicainide from patients who received the intravenous infusion of the drug. For simplicity, the data obtained after 360 minutes postdose were truncated. The inset shows the complete data set. (b) Scatter plots of the observed plasma pilsicainide concentrations versus those predicted by the final population pharmacokinetic (PK) model. The line represents that of unity (y = x). (c) Scatter plots of weighted residuals as a function of the final PK model-predicted plasma drug concentrations. Detailed descriptions of the population PK analysis are given in text.

shape parameter (γ); and the interindividual and residual variabilities in each conduction parameter. Using sequential population PK/PD analysis with the effect compartment model, we successfully accomplished the analysis for PK/PD data with hysteresis. Age and the development of the ST-segment elevation were significant covariates for the K_{e0} of ΔQRS, but clinical implications of these findings remain unclear. While the correlation between the observed and predicted PD responses was not very strong as compared with that between observed and predicted plasma drug concentrations in the PK analysis, prediction of PD responses by the population PD model was considered unbiased. As a typical example, Figure 2 shows the relationship between the observed prolongation of PEQ interval (ΔPEQ) and that predicted by the model (Figure 2a) and the weighted residual plots as a function of predicted drug concentrations (Figure 2b). The data for other PD parameters are shown only in numerical values (Table III). The multivariate analysis revealed that patients developing the drug-induced ST-segment elevation (responders) would have 50% and 40% higher E_{max} values for ΔPEQ (Figure 3) and ΔPQ, respectively, than the nonresponders would ($P < .01$).

DISCUSSION

The present study is the first to perform a population PK/PD analysis of the class IC antiarrhythmic agent pilsicainide in patients with cardiac arrhythmias. The population PK analysis demonstrates that gender and CL_{cr} are independent covariates of the interindividual variability of body-weight-normalized CL of the drug. As shown in Table II, the relationships between CL_{cr} and CL of pilsicainide predicted by the population PK model in male and female patients are clearly different, and female patients have on average 50% lower systemic CL of the drug than do male patients irrespective of body weight and CL_{cr}. There was no statistically significant difference in patients' background between men and women except for body size (ie, total body weight and height). Our data suggest that both gender and CL_{cr} may be important clinical covariates for individualizing doses of pilsicainide during chronic administration. The present study warrants further clinical studies to confirm these findings during long-term administration of the drug.

The finding that the systemic CL of pilsicainide correlates positively with predicted CL_{cr} may be explained by the PK characteristics of the drug. After intravenous administration, >90% of the dose is eliminated primarily via the kidneys into urine in unchanged form in young healthy subjects.[3,8] The mean PK parameters (ie,