Although previous works have suggested that the development of SJS/TEN depends on an immune mechanism involving a drug-dependent cytotoxic cell response against epidermal cells,[5,6] the pathophysiology of SJS/TEN remains largely unknown. Susceptibility to such idiosyncratic reactions is thought to be genetically determined, and familial predisposition to allopurinol-induced SJS/TEN has been reported.[6] Therefore, the exploratory studies for genetic risk factors related to SJS/TEN are needed. A strong association has been observed between allopurinol-induced SCAR and the human lymphocyte antigen (*HLA*) allele B variant (*HLA-B*5801*) in the Han Chinese in Taiwan[7] and in the Thai population.[8] These studies showed that the *HLA-B*5801* allele is present in all patients with allopurinol-induced SCAR (51/51 of Han Chinese and 27/27 of Thai patients) and in only 12–15% of tolerant patients (20/135 and 7/54, respectively). The odds ratio (OR) was 580 (95% confidence interval , 34–9781; $P = 4.7 \times 10^{24}$) for the Han-Chinese data[7] and 348.3 (95% confidence interval, 19.2–6336.9; $P = 1.61 \times 10^{13}$) for the Thai study.[8] Although the association was confirmed in both Caucasian and Japanese subjects,[9,10] the OR in the Han-Chinese and Thai populations were much higher than those in the Caucasian (OR = 80) and Japanese (OR = 40) groups. These reports indicated that *HLA-B*5801* is the valid genetic biomarker for allopurinol-induced SJS/TEN in various ethnic groups, but the mechanisms by which *HLA-B*5801* is specifically involved in allopurinol-induced SJS/TEN progression and the strength of the association showed ethnic differences are unknown.

Currently, genotyping by high-density array scanning of the whole genome allows discovery of previously unsuspected genetic risk factors that influence the pathogenesis of serious adverse drug reactions.[11–13] Genome-wide association studies (GWASs) provide opportunities to uncover polymorphisms that influence susceptibility to allopurinol-induced SJS/TEN free of mechanistic hypotheses. Therefore, in addition to *HLA-B* typing as shown in our previous study,[10] we further conducted a retrospective pharmacogenetic case–control study using whole-genome single nucleotide polymorphism (SNP) data from high-density DNA microarrays in order to identify new and effective genetic biomarkers for allopurinol-related SJS/TEN in Japanese patients.

Materials and methods

Recruitment of study subjects

A total of 141 Japanese SJS/TEN patients from unrelated families were recruited from July 2006 to April 2010 from participating institutes of the Japan Severe Adverse Reactions (JSAR) research group and through a nationwide blood-sampling network system in Japan for SJS/TEN onset patients, operated by the National Institute of Health Sciences.[10] In all, 121 of these patients were diagnosed as defined SJS or TEN by JSAR research group's dermatological experts based on diagnostic criteria[4] that are currently used in Japan. Information was collected using a standardized case report form that includes medical records, co-administrated drug records, disease progress and involvement of systemic complications, as well as SJS/TEN treatment. Among the 141 SJS/TEN patients, 20 were diagnosed as probable SJS due to atypical or mild symptoms. TEN and SJS were defined as mucocutaneous disorders characterized by extensive erythema, blisters, epidermal detachment, erosions, enanthema and high fever. SJS was defined as skin detachment of 10% or less of the body surface area, and TEN as skin detachment of more than 10%, excluding staphylococcal scaled skin syndrome.[5] In all enrolled cases defined as SJS or TEN, allopurinol was regarded as the drug responsible for SJS or TEN if the onset of SJS/TEN symptoms occurred within the first 2 months of allopurinol exposure. For the retrospective pharmacogenetic case–control study, 991 healthy, ethnically matched subjects in the Tokyo metropolitan area were used as the control group. Healthy subjects were used as the control group instead of allopurinol-tolerant patients because the incidence of SJS/TEN is extremely low (0.4–6 per million per year).[3]

The ethics committees of the National Institute of Health Sciences, each participating institute of the JSAR research group and the Japan Pharmacogenomics Data Science Consortium (JPDSC) approved this study. Written informed consent was obtained from all cases and ethnically matched controls.

Whole-genome genotyping of SNPs

Genome-wide genotyping of the 14 allopurinol-related SJS/TEN patients and 991 ethnically matched controls was conducted using the Illumina Human 1M-Duo BeadChip (Illumina, San Diego, CA, USA), which contained 11 63 218 SNPs. SNPs were discarded from case–control association analysis if they exhibited a minor allele frequency <0.001 in the control group (2 37 890 SNPs), a call rate <0.95 for each SNP (32 640 SNPs) or a P-value <0.001 in the test of Hardy–Weinberg equilibrium among controls (2 368 SNPs). These quality control steps removed a total of 2 72 897 SNPs. All samples had a call rate for each microarray above 0.99. Sample duplicates and hidden relatedness were investigated on the basis of pairwise identity-by-state analysis via PLINK;[14] however, there was no duplicate or hidden relatedness in the samples. This quality-control procedure ensured reliable genotyping data.

HLA genotyping and TaqMan genotyping of SNPs on chromosome 6

HLA A, *B* and *Cw* types were determined using sequencing-based methods, as described previously.[10] Representative SNPs of 6p21 (rs2734583, rs3099844, rs9263726 and rs3131643) were re-genotyped using TaqMan SNP Genotyping Assays (Life Technologies, Carlsbad, CA, USA) (ID; C__27465749_10, C__27455402_10, C__30352071_10, C__26778946_20) according to the manufacturer's instruction using 5 ng of genomic DNA. We did not genotype rs9267445 and rs1634776 because TaqMan SNP genotyping assays for these SNPs were not available. Measurement of the linkage disequilibrium (LD) coefficient was performed using

the *HLA* types and 6p21 SNPs of the 141 Japanese SJS/TEN cases and an additional 65 Japanese individuals (non-SJS/TEN patients). The LD coefficient was calculated as previously described.[15,16]

Association analysis

Genome-wide SNPs data from allopurinol-related SJS/TEN cases and ethnically matched controls were used for association analysis using the Fisher's exact test based on the dominant genotype mode and minor allele frequencies of each SNP. Because there are no homozygotes of minor alleles of SNPs, which have significantly related to allopurinol-related SJS/TEN except rs3099844 and rs3131643 in 'Case group', other association analysis models such as trend test (Cochran–Armitage analysis) or recessive model analysis were not applied in this study. All association analyses were carried out with PLINK.[14] *P*-values were corrected for multiple testing according to the Bonferroni's correction. *P*-values $<5.62\times10^{-8}$ were regarded as statistically significant.

Results

Characteristics of study subjects

A total of 14 allopurinol-treated Japanese patients, who were diagnosed with definite SJS/TEN were recruited for the whole-genome association study (IDs 1–14 in Table 1). Patients, IDs 1, 2, 3, 9, 10, 13 and 14 were reported in our previous paper.[10] After the GWAS, an additional four allopurinol-treated Japanese SJS/TEN patients were recruited for *HLA* typing (IDs 15–18). Therefore, a total of 18 allopurinol-treated Japanese SJS/TEN patients participated in the study (Table 1). In all, 12 of 18 patients were male and 6 were female, and the average age was 72.3 ± 10.0 (mean $\pm$ s.d.) years. In all, 12 of 18 cases showed systemic complications of liver and/or renal dysfunction, and most patients had high fever. The average period of SJS/TEN onset after allopurinol treatment was 21.7 ± 11.9 days. Drug-induced lymphocyte stimulation tests were examined in 13 of 18 patients to determine the causative agent; however, in these tests, only two cases (IDs 1 and 5) were positive for allopurinol and only one (ID 16) was positive for oxipurinol, a metabolite of allopurinol. The patient (ID 1) who was positive for the drug-induced lymphocyte stimulation test for allopurinol was also positive for other co-administrated drugs (Table 1). On the other hand, patients who received a patch test showed positive reactions for allopurinol although only two patients were examined (ID 4, 10). The patient who was patch test positive for allopurinol (ID 4) was also patch test positive for other co-administrated drugs (Table 1). Four patients (ID 1, 2, 4 and 14) were co-administrated non-steroidal anti-inflammatory drugs, four (ID 7, 8, 11 and 15) were co-administrated angiotensin II receptor antagonists and three (ID 4, 7 and 17) were co-administrated statin anti-hyperlipemic agents.

Whole-genome association study of major determinants for allopurinol-related SJS/TEN

A total of 14 allopurinol-related SJS/TEN patients (IDs 1–14), who were diagnosed with definite SJS/TEN, and 991 ethnically matched controls, were genotyped with the use of the Illumina Human 1M-Duo BeadChip containing 11 63 218 SNPs. A series of quality-control steps resulted in the elimination of 2 72 897 polymorphisms. For each SNP, Fisher's exact tests were performed to compare the dominant genotype distributions and minor allelic frequencies in the allopurinol-related SJS/TEN patients (the case group) versus those in the ethnically matched healthy control group. The resulting *P*-values were adjusted with the Bonferroni's correction ($P<5.62\times10^{-8}$). The distribution of *P*-values from the Fisher's exact tests (dominant genotype mode) along each chromosome indicated that 21 SNPs were significantly associated with the cases, all of which were located on the chromosome 6: 6p21.3, 6p22.1 and 6p21.1 (Figures 1a and b). The quantile–quantile (Q–Q) plot for the distribution of *P*-values showed that observed *P*-values matched the expected *P*-values over the range of $0<-\log_{10}(p)<4.0$ (Figure 2). A departure was observed at the extreme tail ($-\log_{10}(p)>4.0$) of the distribution of test statistics for the allopurinol-related Japanese SJS/TEN, suggesting that the identified associations are likely due to true variants rather than potential biases such as genotyping error. These SNPs, with their associated genes, are described in Table 2. As is observed in all SNPs in Table 2, minor allele frequencies in the controls were quite small, ranging around 0.5–0.6%. The genotypic distributions of the case and control groups are identical among groups with the same *P*-value, suggesting that these SNPs might be linked. These SNPs also have ORs that are much higher than the ORs of SNPs commonly observed in sporadic cancer and other complex diseases, suggesting they are of higher penetrance. For example, the most significant SNPs (rs2734583, rs3094011 and GA005234) had an OR of 66.8 (95% confidence interval, 19.8–225.0), and the twentieth most significant SNPs (rs9263827 and rs1634776) had an OR of 60.9 (95% confidence interval, 18.3–202.5). Most SNPs in Table 2 are associated with known or predicted genes; of these, 13 are in known genes. Three SNPs (rs17190526, rs9263726 and rs2233945) were found in *PSORS1C1* (psoriasis susceptibility 1 candidate 1), which is considered as one of the potential psoriasis genes.[17–19] The *CCHCR1* (coiled coil α helical rod protein 1), which is a regulator of keratinocyte proliferation or differentiation and is over-expressed in keratinocytes in psoriatic lesions,[20–23] contained four SNPs (rs9263745, rs130077, rs9263781 and rs9263785). *HCP5* (HLA complex P5), which is involved in hypersensitivity to abacavir,[24–26] had three SNPs (rs3094011, rs3099844 and rs31431643). *TCF19* (transcription factor 19), which is a potential trans-activating factor that might play an important role in the transcription of genes required for the later stages of cell cycle progression,[27] contained two SNPs (rs9263794 and rs10448701). Two SNPs (rs9263796 and rs9263800) were also found in *POU5F1* (POU class 5 homeobox; alternative names for Oct4). *BAT1* (HLA-B

Table 1 Summary of clinical characteristics of Japanese patients with allopurinol-related Stevens–Johnson syndrome or toxic epidermal necrolysis

								Co-administered drugs	
Patient ID[a]	*ADR type*	*Sex/age (years)*	*Highest BT (°C)*	*Total area of blistering skin (%)*	*Systemic complications*	*DLST to allopurinol (PT)*	*Period of onset (days) by allopurinol*	*Drug name*	*DLST result/period of onset*
1	SJS	F/53	38.1	0.5	liver dysfunction renal dysfunction	+	26	loxoprofen clarithromycin	+/9 days +/26 days
2	TEN	M/58	37.1	15	neutropenia liver dysfunction	–	ca 10 days	loxoprofen levofloxacin	–/1 day –/1 day
3	SJS	M/77	unknown	unknown	none	not tested	16	none	
4	TEN	F/72	>37	20	none	–(PT+)	16	pitavastatin lansoprazole salicylamide, acetaminophen, caffeine, promethazine, methylenedisalicylate serrapeptase loxoprofen acetaminophen	–/16 days –/179 days –(PT+)/8 days –/1 day –/8 days (PT+)/8 days
5	TEN	M/82	39	35	none	+	52	none	
6	SJS	M/67	1	1	liver dysfunction	not tested	14	none	
7	SJS	M/76	38.8	unknown	GI tract disturbance liver dysfunction renal dysfunction	not tested	<26 days	losartan furosemide carbon atorvastatin	not tested/8 days not tested/3 days not tested/7 days not tested/8 days
8	SJS	M/83	>38	10	renal dysfunction	–	20	amlodipine olmesartan medoxomil	not tested/very long not tested/very long
9	TEN	M/75	>38	20	neutropenia liver dysfunction renal dysfunction	–	6	none	
10	SJS	M/75	38.4	6	neutropenia liver dysfunction renal dysfunction	–(PT+)	14	none	
11	SJS	M/74	37.8	8	neutropenia liver dysfunction renal dysfunction	–	38	cefazolin Furosemide Sodium polystyrene sulfonate olmesartan medoxomil	not tested/1 day not tested/53 day not tested/51 day not tested/59 day
12	SJS	M/67	38.9	2	liver dysfunction	not tested	17	none	
13	SJS	F/81	39.2	0.5	renal dysfunction	–	28	spironolactone	–/24 days
14	SJS	M/83	39	0	respiratory involvement	–	29	diclofenac	–/1 day
15	TEN	F/73	38	10	liver dysfunction renal dysfunction	–	27	valsartan epoetin β	–/18 days –/2 days
16	SJS	M/53	40	5	liver dysfunction	–(oxipurinol +)	19	none	
17	SJS	F/86	38	0	liver dysfunction renal dysfunction	–	30	rosuvastatin	–/43 days
18	TEN	F/66	37.8	15	none	not tested	2	none	

Abbreviations: ADR, adverse drug reaction; BT, body temperature; DLST; drug-induced lymphocyte stimulation test; F, female; M, male; PT, patch test; SJS, Stevens–Johnson syndrome; TEN, toxic epidermal necrolysis.

[a]Patients ID 1–14 were applied for whole genome analysis. ID 1–18 were for the *HLA* typing and the analysis of linkage disequilibrium.

Patients IDs 1, 2, 3, 9, 10, 13, and 14 were reported in our previous paper.[10]

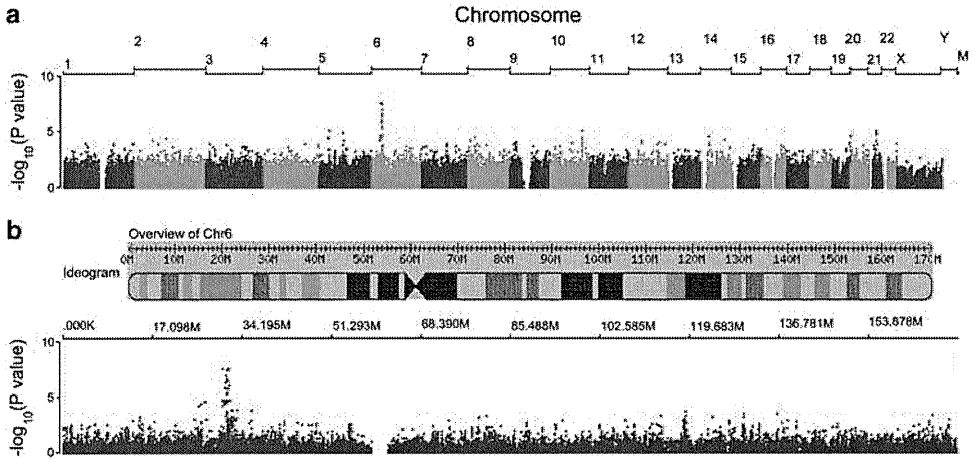

Figure 1 Genome-wide association study of allopurinol-related Stevens–Johnson syndrome or toxic epidermal necrolysis. Each dot represents a single nucleotide polymorphism (SNP). The *x* axis: the position of the SNP on chromosomes. The *y* axis: the $-\log_{10}$ of Fisher's exact test *P*-values (dominant genotype mode) of the SNP in the case–control association study. SNPs with *P*-values $<5.62 \times 10^{-8}$ are highlighted in red. (**a**) Whole genome. (**b**) Chromosome 6.

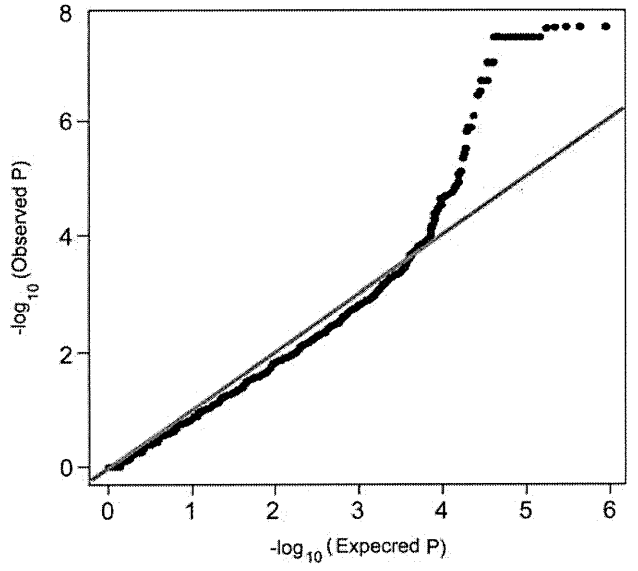

Figure 2 Quantile–quantile plot of Fisher's exact test statistics obtained from the genome-wide association study for allopurinol-related Stevens–Johnson syndrome or toxic epidermal necrolysis under dominant genotype mode. The solid red line represents the null model where observed Fisher's exact test values match the expected values. The dots represent observed versus the expected values from the case–control study.

associated transcript 1) and *PSORS1C3* each carried one SNP (rs2734583 and rs9263827). The SNPs, rs1634776 and rs4084090, were located in more than 10 kb away from the *HLA-B* and *HLA-C* genes, respectively. Two pseudo genes, *MICC* (major histocompatibility complex class I polypeptide-related sequence) and *PPIAP9* (peptidylprolyl isomerase A (cyclophilin A) pseudogene 9), had one SNP each (GA005234 and rs9267445). Previous report using Han-Chinese patients with allopurinol-induced SCAR indicated rs3117583 of *BAT3*, rs1150793 of *MSH5* and rs2855804 of *MICB*, which are located in *HLA* region, showed significant *P*-values ($P<1 \times 10^{-7}$).[7] In this study using Japanese patients, both rs3117583 and rs1150793 showed $P=6.34 \times 10^{-3}$ (allele frequency mode) and $P=6.14 \times 10^{-3}$ (dominant genotype mode). There was no data of rs2855804 in the Illumina Human 1M-Duo BeadChip.

HLA types of allopurinol-related SJS/TEN patients

Classical class I *HLA* types (*A*, *B* and *Cw*) of allopurinol-related SJS/TEN patients were determined because the *HLA-B*5801* type is associated with allopurinol-related SCARs in Han Chinese,[7] Caucasians[9] and Japanese[10] (Table 3). In this analysis, four patients with allopurinol-related SJS/TEN (IDs 15–18), who were recruited after BeadChip analysis, joined the case group (total of 18 allopurinol-related SJS/TEN patients). Eight cases of *HLA-A*3303* (allele frequency = 22.2%), 10 cases of *HLA-B*5801* (allele frequency = 27.8%) and 10 cases of *HLA-Cw*0302* (allele frequency = 27.8%) were found in 18 allopurinol-related SJS/TEN patients (Table 3). By comparison, the allelic frequencies of *HLA-A*3303*, *HLA-B*5801* and *HLA-Cw*0302* were 7.9%, 0.6% and 0%, respectively in Japanese general population (Tables 4a–c). The OR of *HLA-A*3303* was calculated as 3.32 (Table 4a). The OR of *HLA-B*5801* was calculated as 62.8 (Table 4b), which was a little larger than the previously reported OR in Japanese patients.[10] *HLA-Cw*0302* also showed significant association with allopurinol-related SJS/TEN (Table 4c). *HLA-A*3303* and *HLA-Cw*0302* are in LD with *HLA-B*5801* in the Japanese although the general frequency of *HLA-A*3303* is higher than other two types. Other *HLA-A*, *B* and *Cw* types, which were not listed in Tables 4a–c, showed very low frequencies in the general Japanese population, or were not found in 18 allopurinol-related SJS/TEN patients.

Table 2 The association of single nucleotide polymorphism with allopurinol-related Japanese patients with Stevens–Johnson syndrome or toxic epidermal necrolysis

							Dominant genotype mode		Allelic frequency mode	
Order	*SNP*	*Chromosome*	*Closest gene*	*Distance to gene (bp)*	*Case*[a]	*Control*[a]	P	*Odds ratio (95% CI)*	P	*MAF (%)*
1	rs2734583	6p21.3	*BAT1*	0	0/6/8	0/11/980	2.44×10^{-8}	66.8 (19.8–225.0)	4.62×10^{-8}	0.55
1	rs3094011	6p21.3	*HCP5*	6553	0/6/8	0/11/980	2.44×10^{-8}	66.8 (19.8–225.0)	4.62×10^{-8}	0.55
1	GA005234	6p22.1	*MICC*	0	0/6/8	0/11/980	2.44×10^{-8}	66.8 (19.8–225.0)	4.62×10^{-8}	0.55
4	rs3099844	6p21.3	*HCP5*	3693	1/5/8	0/11/978	2.47×10^{-8}	66.7 (19.8–224.5)	1.33×10^{-9}	0.56
5	rs9267445	6p21.1	*PPIAP9*	3776	0/6/8	0/11/971	2.58×10^{-8}	66.2 (19.7–222.9)	4.87×10^{-8}	0.56
6	rs17190526	6p21.3	*PSORS1C1*	−446	0/6/8	0/12/979	3.64×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs9263726	6p21.3	*PSORS1C1*	0	0/6/8	0/12/979	3.64×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs2233945	6p21.3	*PSORS1C1*	0	0/6/8	0/12/979	3.64×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs9263733	6p21.3	*POLR2LP*	139	0/6/8	0/12/979	3.64×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs9263745	6p21.3	*CCHCR1*	0	0/6/8	0/12/979	3.64×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs130077	6p21.3	*CCHCR1*	0	0/6/8	0/12/979	2.44×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs9263781	6p21.3	*CCHCR1*	0	0/6/8	0/12/979	2.44×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs9263785	6p21.3	*CCHCR1*	0	0/6/8	0/12/979	2.44×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs9263794	6p21.3	*TCF19*	0	0/6/8	0/12/979	2.47×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs1044870	6p21.3	*TCF19*	0	0/6/8	0/12/979	2.58×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs9263796	6p21.3	*POU5F1*	0	0/6/8	0/12/979	3.64×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs9263800	6p21.3	*POU5F1*	0	0/6/8	0/12/979	3.64×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
6	rs4084090	6p21.3	*HLA-C*	17691	0/6/8	0/12/979	3.64×10^{-8}	61.2 (18.4–203.5)	6.87×10^{-8}	0.61
19	rs3131643	6p21.3	*HCP5*	0	1/5/8	0/12/977	3.68×10^{-8}	61.1 (18.4–203.1)	2.08×10^{-9}	0.61
20	rs9263827	6p21.3	*PSORS1C3*	−3369	0/6/8	0/12/974	3.75×10^{-8}	60.9 (18.3–202.5)	7.07×10^{-8}	0.61
20	rs1634776	6p21.3	*HLA-B*	12661	0/6/8	0/12/974	3.75×10^{-8}	60.9 (18.3–202.5)	7.07×10^{-8}	0.61

Abbreviations: CI, confidence interval; MAF; minor allelic frequency; SNP, ingle nucleotide polymorphism.
[a]Number of subjects in minor homo/hetero/major homo.

Table 3 *HLA* types and representative genotypes in 6p21 of allopurinol-related Japanese patients with Stevens–Johnson syndrome or toxic epidermal necrolysis

ID	HLA-A		HLA-B		HLA-Cw		*rs2734583*	*rs3099844*	*rs9267445*	*rs9263726*	*rs3131643*	*rs1634776*
1	2402	3303	4002	**5801**	0302	0304	T/**C**	C/**A**	G/**C**	G/A	C/T	G/A
2	2402	3101	1501	5601	0303	0401	T/T	C/C	G/G	G/G	C/C	G/G
3	2402	3101	5201	**5801**	0302	1202	T/**C**	C/**A**	G/**C**	G/A	C/T	G/A
4	1101	1101	4801	**5801**	0302	0803	T/**C**	**A**/**A**	G/**C**	G/A	**T**/**T**	G/A
5	2402	2602	4006	5101	0801	1402	T/T	C/C	G/G	G/G	C/C	G/G
6	0201	1101	1518	3501	0401	0801	T/T	C/C	G/G	G/G	C/C	G/G
7	2402	3303	5201	**5801**	0302	1202	T/**C**	C/**A**	G/**C**	G/A	C/T	G/A
8	0201	2402	1527	4003	0304	0401	T/T	C/C	G/G	G/G	C/C	G/G
9	2402	2402	3501	5201	0303	1202	T/T	C/C	G/G	G/G	C/C	G/G
10	0210	1101	4002	4006	0401	0801	T/T	C/C	G/G	G/G	C/C	G/G
11	0207	2402	4601	5101	0102	1402	T/T	C/C	G/G	G/G	C/C	G/G
12	2402	3101	3901	4001	0304	0702	T/T	C/C	G/G	G/G	C/C	G/G
13	0207	3303	4601	**5801**	0102	0302	T/**C**	C/**A**	G/**C**	G/A	C/T	G/A
14	3101	3303	3901	**5801**	0302	0702	T/**C**	C/**A**	G/**C**	G/A	C/T	G/A
15	2402	3303	5101	**5801**	0302	1402	T/**C**	C/**A**	NA	G/A	**T**/**T**	NA
16	0201	3303	3802	**5801**	0302	0702	T/**C**	C/**A**	NA	G/A	**T**/**T**	NA
17	2402	3303	0702	**5801**	0302	0702	T/**C**	C/**A**	NA	G/A	C/T	NA
18	2402	3303	5101	**5801**	0302	0304	T/**C**	C/**A**	NA	G/A	**T**/**T**	NA

Abbreviations: HLA, human leukocyte antigen; NA, not available.
Single nucleotide polymorphisms data of rs2734583, rs3099844, rs9263726 and rs3131643 are from BeadChip analysis and TaqMan genotyping analysis. Single nucleotide polymorphisms data of rs9267445 and rs1634776 are from BeadChip analysis.
Underlines of HLA types mean that these types are in linkage disequilibrium. HLA-B*5801s are expressed by bold types.
Bold types of the nucleotide mean the variant allele.

Table 4a Association between *HLA-A* alleles and allopurinol-induced Stevens–Johnson syndrome or toxic epidermal necrolysis

HLA-A allele	*Number of alleles detected (allele frequency)*		P	*Odds ratio (95% CI)*
	Case, n = 36 (%)	*General population control (n = 986)[a] (%)*		
0201	3 (8.3)	10.9	0.7895	
0206	0 (0)	10.4	0.0426	
0207	2 (5.6)	3.4	0.3650	
0210	1 (2.8)	0.1	0.0692	
1101	4 (11.1)	8.1	0.5299	
2402	13 (36.1)	35.6	1.000	1.02 (0.51–2.04)
2601	0 (0)	9.8	0.0417	
2602	1 (2.8)	2.2	0.5657	
3101	4 (11.1)	7.7	0.5195	
3303	8 (22.2)	7.9	0.0077	3.32 (1.46–7.54)

Abbreviations: CI, confidence interval; HLA, human leukocyte antigen.
We listed the *HLA-A* types of which the allele frequencies in the Japanese population are more than 9% or which were detected in this study.
[a]General population control data are cited from Tanaka *et al.*[40]

Table 4b Association between *HLA-B* alleles and allopurinol-induced Stevens–Johnson syndrome or toxic epidermal necrolysis

HLA-B allele	*Number of alleles detected (allele frequency)*		P	*Odds ratio (95% CI)*
	Case, n = 36 (%)	*General population control (n = 986)[a] (%)*		
0702	1 (2.8)	5.2	1.000	
1501	1 (2.8)	7.2	0.5076	
1518	1 (2.8)	0.9	0.3025	
1527	1 (2.8)	0	0.0352	
3501	2 (5.6)	8.6	0.7621	
3802	1 (2.8)	0.3	0.1338	
3901	2 (5.6)	4.0	0.6520	
4001	1 (2.8)	5.1	1.0000	
4002	2 (5.6)	8.2	0.7620	
4003	1 (2.8)	1.1	0.3512	
4006	2 (5.6)	5.3	0.7150	
4403	0 (0)	6.9	0.1648	
4601	2 (5.6)	3.8	0.6441	
4801	1 (2.8)	2.7	1.0000	
5101	4 (11.1)	7.9	0.5244	
5201	3 (8.3)	13.7	0.4624	
5401	0 (0)	6.5	0.1620	
5601	1 (2.8)	1.0	0.3273	
5801	10 (27.8)	0.6	5.388×10^{-12}	62.8 (21.2–185.8)

Abbreviations: CI, confidence interval; HLA, human leukocyte antigen.
We listed the *HLA-B* types of which the allele frequencies in the Japanese population are more than 6.5% or which were detected in this study.
[a]General population control data are cited from Tanaka *et al.*[40]

*LD of HLA-B*5801 with SNPs on chromosome 6*

We compared the genotypic distributions of six SNPs, which were significantly associated with SJS/TEN (Table 2), with *HLA* types because these SNPs are located near the *HLA-B* gene. These 6 SNPs listed in Table 3 represent 21 SNPs in Table 2 because the other 15 SNPs are in absolute LD with 1 of the 6 SNPs. Representative six variants of the significant SNPs on chromosome 6 were found in all of the SJS/TEN patients who carried the *HLA-B*5801* (10 patients) (Table 3). Therefore, in order to evaluate LD in the Japanese

Table 4c Association between *HLA-Cw* alleles and allopurinol-induced Stevens–Johnson syndrome or toxic epidermal necrolysis

HLA-Cw allele	*Number of alleles detected (allele frequency)*		P	*Odds ratio (95% CI)*
	Case, n = 36 (%)	*General population control (n = 234)[a] (%)*		
0102	2 (5.6)	17.0	0.0859	
0302	10 (27.8)	0	5.303×10^{-10}	
0303	2 (5.6)	7.8	1.000	
0304	4 (11.1)	11.3	1.000	
0401	4 (11.1)	6.5	0.2961	
0702	4 (11.1)	11.3	1.000	
0801	3 (8.3)	10.9	0.7777	
0803	1 (2.8)	2.6	1.000	
1202	3 (8.3)	10.4	1.000	
1402	3 (8.3)	5.7	0.4559	
1403	0 (0)	12.2	0.0192	

Abbreviations: CI, confidence interval; HLA, human leukocyte antigen.
We listed the *HLA-Cw* types of which the allele frequencies in the Japanese population are more than 10% or which were detected in this study.
[a]General population control data are cited from Tokunaga *et al.*[41]

Table 5 The linkage disequilibrium between *HLA* types and representative single nucleotide polymorphisms on 6p21 of 206 Japanese individuals

HLA	*rs3099844*	*rs3131643*	*rs2734583*	*rs9267445*	*rs9263726*	*rs1634776*
A	0.821	0.621	0.835	0.798	0.847	0.803
B	0.973	0.873	1.000	1.000	1.000	0.996
Cw	0.984	0.773	1.000	1.000	1.000	0.909

Abbreviation: HLA, human leukocyte antigen.
Data are expressed in D'.

Table 6 The linkage disequilibrium between representative single nucleotide polymorphisms on 6p21 and *HLA-B*5801* of 206 Japanese individuals

SNP	D'	r^2
rs3099844	0.930	0.866
rs3131643	0.929	0.674
rs2734583	1.000	0.931
rs9267445	1.000	0.896
rs9263726	1.000	1.000
rs1634776	1.000	0.905

Abbreviation: SNP, single nucleotide polymorphism.

population, LD coefficients (D') were calculated between classical class 1 *HLA* types and six representative SNPs at 6p21, using the *HLA*-type and SNPs genotype data of 206 Japanese individuals, including 141 SJS/TEN cases and an additional 65 non-SJS/TEN Japanese subjects. As shown in Tables 5 and 6 representative SNPs on chromosome 6 showed LD for the *HLAs*. In particular, three SNPs (rs2734583, rs9267445 and rs9263726) showed a strong linkage with *HLA-B* and *Cw* alleles (Table 5). LD between six representative SNPs in 6p21 and *HLA-B*5801* are shown in Table 6. A novel observation was the absolute LD ($D' = 1$, $r^2 = 1$) between rs9263726 in *PSORS1C1* and the *HLA-B*5801* allele.

Discussion

In order to explore new genetic biomarkers associated with the occurrence of allopurinol-related SJS/TEN Japanese patients, we conducted a GWAS using 890321 SNPs from patients with allopurinol-related SJS/TEN and an ethnically matched control group. The GWAS data indicated that most SNPs significantly associated with allopurinol-related SJS/TEN are located on or close to genes that overlap the 6p21 region, especially the genes neighboring *HLA-B*. There was no significantly associated SNP in any other region of the genome (Figures 1 and 2 and Table 2), indicating that the 6p21 region has the most important role in the progress of allopurinol-related SJS/TEN. We expected to find SJS/TEN-associated SNPs, which are unrelated to *HLA-B*5801* from this GWAS study because the association of *HLA-B*5801* with SJS/TEN is incomplete (10/18) in Japanese patients in contrast to Han Chinese[7] and Thai patients.[8] However, most

of significant SNPs were closely linked with *HLA-B*5801* (Table 6). Previous studies have indicated that a SNP (rs2395029) in the *HCP5*, which is on 6p21.3, is strongly associated with human immunodeficiency virus-1 set points,[28–30] abacavir-induced hypersensitivity[24–26] and flucloxacillin-induced liver injury.[31] This SNP is in strong LD with *HLA-B*5701* in Caucasians.[25] Another SNP in 6p21 in *PSORS1C1*, a psoriasis-susceptibility candidate gene, was related with psoriasis in Swedish and Canadian populations[17,18] and exhibits LD with *HLA-Cw*0602* in Canadian populations.[18] These reports suggest that SNPs located in 6p21 link with a specific type of classical class I *HLA* that could be an alternative biomarker for the physiological phenomenon. Therefore, we examined the LD between these SNPs, shown in Table 2, and *HLA-B*5801*, which has been regarded as a genetic biomarker of SJS/TEN not only in Han Chinese,[7] but also in Caucasians[9] and Japanese.[10] We found that all of the Japanese patients with the allopurinol-related SJS/TEN who had the *HLA-B*5801* (10 patients) also had variant SNPs of genes that are located in 6p21, including *BAT1*, *HCP5*, *PPIAP9*, *PSORS1C1* and *HLA-B* (Table 3). The analysis of the LD coefficients between SNPs located in 6p21 and *HLA* types in the Japanese population indicated that these SNPs are in strong LD with *HLA* types (Table 5), and an absolute LD between rs9263726 in *PSORS1C1* and *HLA-B*5801* was observed in the Japanese population (Table 6). These results mean that all subjects (14 individuals including 10 with allopurinol-related SJS/TEN) who carry *HLA-B*5801* are in complete accord with all subjects with minor A allele of rs9263726 in the Japanese population. Therefore, rs9263726 in *PSORS1C1* is an alternative biomarker for *HLA-B*5801* in the Japanese population. Conventional genotyping of rs9263726 based on allelic discrimination offers several advantages over *HLA-B* typing, which is determined by genotyping of several SNPs forming the *HLA-B*5801* haplotype. Various broadly used technologies (for example, TaqMan genotyping) allow the standardized identification of two distinct alleles in one reaction tube, limiting the risk of contamination and allowing high-throughput genotyping with high sensitivity and specificity. In addition, the test is largely independent of both the performance of and interpretation by laboratory personnel. SNP genotyping is also less time consuming and cheaper than sequence-based *HLA* typing, and it does not require specialized laboratories. Therefore, the easy detection of these SNPs has a practical and economical advantage in clinical application for predicting the onset of allopurinol-related SJS/TEN. Although the previous report revealed that three SNPs in *HLA* region strongly associated with allopurinol-related SCAR in Han Chinese,[7] the two SNPs analyzed by the Illumina Human 1M-DUO BeadChip showed only weak association in the Japanese. This ethnic difference might be due to the difference of LD.

The functional analysis of genes that carry these SNPs—including *HCP5*, *BAT1*, *PSORS1C1*, *CCHCR1*, *TCF19* and *POU5F1*—in the pathogenesis of allopurinol-related SJS/TEN might be useful for determining their relevance. *CCHCR1* is a regulator of keratinocyte proliferation or differentiation and is overexpressed in keratinocytes in psoriatic lesions.[20–23] *TCF19* is a potential trans-activating factor that could play an important role in the transcription of genes required for the later stages of cell cycle progression.[27] Possible psoriasis candidate genes near *HLA-B* include *PSORS1C1*,[17–19] *CCHCR1*,[22,23] and *POU5F1*.[32,33] Mutations in *BAT1* may be associated with rheumatoid arthritis.[34–36] *HCP5* encodes an endogenous retroviral element mainly that is expressed in immune cells and there is evidence that the SNP in this gene is protective against human immunodeficiency virus-1 infection.[37–39] The functions and relevance of these genes suggest that the pathogenesis of allopurinol-related SJS/TEN might involve not only an immune system disorder, but also processes of cell proliferation and differentiation.

In conclusion, the results of this GWAS of allopurinol-related SJS/TEN in Japanese patients show that SNPs in genes located in 6p21, which are in LD with *HLA-B*5801*, are strongly associated with the cutaneous adverse reaction. Therefore, these SNPs, especially rs9263726, prove to be predictors for allopurinol-related SJS/TEN in Japanese, and their genes might be involved in the pathogenesis of allopurinol-related SJS/TEN. The OR of rs9263726 is extremely high from this case–control study and the typing cost of SNP is much cheaper than that of *HLA* typing. Moreover, the SJS/TEN has a very severe adverse reaction of allopurinol, which is high mortality. Therefore, we believe that the screening of rs9263726 genotype before allopurinol administration is necessary to prevent SJS/TEN in allopurinol-treated Japanese patients, although its allele frequency is very low in the Japanese. Association analyses of other ethnic populations are needed for confirming and comparing the results obtained in this study. *In vitro* functional studies of these genes are also necessary for identification of the physiological and molecular pathways leading to allopurinol-related SJS/TEN.

Conflict of interest

The authors declare no conflict of interest except one member of JPDSC, Mitsubishi Tanabe Pharma, which is a distributor of allopurinol in Japan.

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Appendix

Japan Pharmacogenomics Data Science Consortium (JPDSC)

The Japan Pharmacogenomics Data Science Consortium is composed of Astellas Pharma, Otsuka Pharmaceutical, Daiichi Sankyo, Taisho Pharmaceutical, Takeda Pharmaceutical and Mitsubishi Tanabe Pharma, and is chaired by Ichiro Nakaoka (Takeda Pharmaceutical).

Effect of 2 Weeks' Consumption of Pomegranate Juice on the Pharmacokinetics of a Single Dose of Midazolam: An Open-Label, Randomized, Single-Center, 2-Period Crossover Study in Healthy Japanese Volunteers

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ABSTRACT

Background: It has been reported that pomegranate juice significantly increased the AUC of orally administered carbamazepine in rats, which suggests that pomegranate may inhibit the cytochrome P450 3A (CYP3A)–mediated carbamazepine metabolism.

Objective: The aim of the present study was to clarify the effect of repeated consumption of pomegranate juice on CYP3A activity by assessing the pharmacokinetics of midazolam, a typical CYP3A probe drug, and its metabolites in healthy volunteers.

Methods: An open-label, randomized, single-center, 2-period crossover study was conducted on healthy Japanese volunteers. Each subject received 200 mL of pomegranate juice twice daily for 2 weeks. On day 14, they were administered 15 μg/kg midazolam orally with either pomegranate juice or water. Plasma concentrations and urinary excretions of midazolam, 1′-hydroxymidazolam, and 4-hydroxymidazolam were determined up to 24 hours using LC/MS/MS and analyzed by a noncompartmental method.

Results: Sixteen subjects (11 men and 5 women) were enrolled and completed the study. The mean (SD) age was 24.1 (4.8) years (range 22–40), mean body weight was 62.9 (8.8) kg (range 45.6–79.9). Differences in the mean $AUC_{0-\infty}$ were 12.7 (4.4) and 14.2 (6.6) ng/mL/h in pomegranate juice and control groups, respectively (geometric mean ratio: 1.02 [95% CI, 0.95–1.09]; $P = 0.40$). Differences in C_{max} for midazolam did not reach the level of statistical significance (5.1 [1.7] vs 5.0 [2.0] ng/mL, geometric mean ratio: 0.95 [95% CI, 0.79–1.11]; $P = 0.68$). Excretions of 1′-hydroxymidazolam ($P = 0.34$) and 4-hydroxymidazolam ($P = 0.32$) were not significantly altered by ingestion of pomegranate juice.

Conclusion: In this small Japanese adult volunteer population receiving single subtherapeutic doses of midazolam, 2 weeks' consumption of pomegranate juice did not significantly alter the pharmacokinetic profile of midazolam compared with that of the control. Protocol identifier: UMIN000004459. (*Clin Ther.* 2011;33:246–252)

Key words: CYP3A, midazolam, pharmacokinetics, pomegranate juice.

INTRODUCTION

Food–drug interactions, especially fruit juice–drug interactions, have received considerable attention for years.[1–3] Several fruit juices, including apple juice, grapefruit juice, and orange juice, were reported to induce clinically significant drug interactions.[4–6] Most of these interactions are involved in the inhibition of drug-metabolizing enzymes such as cytochrome P450 (CYP) enzymes, mainly CYP3A, and/or drug transporters such as P-glycoprotein and organic anion-transporting polypeptides (OATP).[2,7] The inhibition of these enzymes in the intestine leads to elevated systemic exposure of substrate drugs and subsequently increases the risk of adverse drug reactions. In particular, drug interactions with grapefruit juice have been

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well studied in clinical settings. For example, even a single intake of grapefruit juice resulted in a marked increase in the oral bioavailability of coadministered drugs through an irreversible inhibition of intestinal CYP3A or OATP1A2.[8,9] Furthermore, pharmacokinetic alterations induced by the intake of grapefruit juice frequently affect pharmacodynamic actions.[8,10,11] For other fruit juices, such as cranberry juice, grape juice, and pomegranate juice, however, less information is available at present on clinically relevant interactions (searching MEDLINE and Cochrane databases[3]). Thus, there is a further need to evaluate the possibility of fruit juice–drug interactions in humans.

Pomegranate (*Punica granatum L.*) is an edible fruit that is consumed fresh and in processed forms, such as juice, wines, and extracts. Pomegranate has been used for centuries in ancient cultures for its medicinal purposes, and numerous studies have suggested its health effects.[12–14] Pomegranate juice contains higher levels of polyphenolic compounds than other fruit juices, such as grapefruit, orange, apple, and cranberry juice.[13] The principle polyphenols include ellagitannins and anthocyanins, which are believed to contribute significantly to antioxidative and antiinflammatory actions.[14,15] In addition, pomegranate may also decrease the risk of cardiovascular disease, suppresses prostate and breast cancers, and even augment the effect of HIV treatment regimens.[13,16,17] Perhaps in response to these reported beneficial effects, pomegranate juice has become increasingly popular as a natural antioxidant, being consumed at a rate of 500,000 liters per week in the United Kingdom.[18] Thus, it is also predicted that the number of patients who receive drugs and consume pomegranate juice concomitantly will increase in the future.

It was reported that pomegranate juice significantly increased the area under the concentration-time curve (AUC) of orally administered carbamazepine in rats, suggesting that pomegranate might inhibit the CYP3A-mediated carbamazepine metabolism.[19] Pomegranate juice also altered the pharmacokinetics of tolbutamide, which could be metabolized by CYP2C9 in rats.[20] In addition, pomegranate juice consumption decreased total hepatic CYP content as well as the expression of CYP1A2 and CYP3A in mice.[21] In contrast to the results from animal studies, Farkas et al reported that ingestion of a single bolus of pomegranate juice did not alter the clearance of orally and intravenously administered midazolam, an in vivo CYP3A probe, in healthy male volunteers, indicating the species differences between rodents and humans in the inhibitory effects of pomegranate juice.[22] However, whether the repeated ingestion of pomegranate juice could alter the pharmacokinetic profile of CYP3A substrate drugs is still unknown.

The aim of the present study was to assess the effect of repeated consumption of pomegranate juice on the pharmacokinetic profile of midazolam. After continuous ingestion of pomegranate juice for 2 weeks, midazolam was administered orally, and then the AUC and other pharmacokinetic parameters of midazolam and its metabolites were estimated in healthy volunteers. Urinary excretions of 1′-OH midazolam and 4-OH midazolam were also monitored up to 24 hours after midazolam administration.

METHODS

Subjects

Sixteen healthy Japanese volunteers (11 men and 5 women; age range, 22–40 years; mean [SD] body weight, 63.0 [8.8] kg, ranging from 45.6 to 79.9 kg) were enrolled in the study after giving written informed consent. Subjects were in good health as indicated by medical history; routine physical examination; and vital signs, such as heart rate and blood pressure and clinical laboratory data, including white blood cells, red blood cells, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelets, total protein, albumin, glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, lactate dehydrogenase, alkaline phosphatase, γ-glutamyltransferase, total bilirubin, direct bilirubin, creatinine, blood urea nitrogen, uric acid, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglyceride, Na, K, Cl, and blood sugar, with coefficient of variation values for all items being less than 0.8%. They were nonsmokers and refrained from taking prescription or nonprescription drugs and dietary or herbal supplements from 1 week before the study. The volunteers were randomly divided into 2 groups of 8 to facilitate the crossover phase. The study was approved by the Ethics Committee of Hamamatsu University School of Medicine (Hamamatsu, Japan) and was conducted according to principles of the Declaration of Helsinki.

Study Design

In an open-label, randomized, crossover trial with a washout of at least 1 week, the subjects ingested 200 mL of normal-strength, commercially available pomegranate juice (Kikkoman, Chiba, Japan) or water twice a day for 14 days in each phase. On day 14 of each phase, subjects received midazolam* orally at a dose of 15 μg/kg with 200 mL of pomegranate juice or water in the morning. The subjects fasted overnight before the administration of midazolam and had a standard meal at 1 and 4 hours later, as described previously.[23] Tolerability was assessed by investigator monitoring for adverse events before the administration and every 2 hours after the administration of midazolam. Blood samples (5 mL) were obtained 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, and 24 hours following midazolam administration through an indwelling catheter placed in an antecubital vein. Before blood collection, 2 mL of blood was discarded, and after blood sampling, 1.5 mL of heparin (100 units/mL) was used to flush lines. Plasma samples were isolated from blood by centrifugation (1997 × *g*, 4°C) for 10 minutes. Urine was collected cumulatively up to 24 hours after the administration of midazolam. Plasma and urine samples were stored at −80°C until analysis.

Determination of Midazolam and its Metabolites Concentrations

The concentrations of midazolam, 1′-hydroxymidazolam, and 4-hydroxymidazolam in plasma and urine were analyzed using high-performance liquid chromatography/tandem mass spectrometry as described previously.[24] In brief, urine samples were preincubated with β-glucuronidase (500 U) in 1 mL of 50 mM sodium acetate buffer (pH 5.0) at 37°C for 24 hours. Plasma or urine (0.5 mL) samples containing nitrazepam (10 ng) as an internal standard were added to 0.5 mL of disodium phosphate buffer (10 mM, pH 9.1) and 5 mL of diethyl ether–methylene chloride (7:3, v/v). After shaking for 10 minutes, the organic phase was evaporated under a gentle stream of nitrogen gas at 40°C. The residue was reconstituted in 50 μL of mobile phase (methanol-10 mM ammonium acetate, 60:40, v/v). HPLC analysis was performed using an analytical column (Symmetry C_{18}, 5 μm, 2.1 mm × 150 mm; Waters, Milford, Massachusetts) with an isocratic mobile phase delivered at a flow rate of 0.2 mL/min at room temperature. Positive ion electrospray ionization was measured using a tandem mass spectrometer operated with the following mass transitions: *m/z*326→*m/z*291 for midazolam, *m/z*342→*m/z*324 for 1′-hydroxymidazolam, and *m/z*343→*m/z*123 for 4-hydroxymidazolam. The limit of quantification was 0.1 ng/mL for each compound, and the method was linear from 0.1 to 100 ng/mL. The intra-assay CV values were <8.1% for midazolam, <10.9% for 1′-hydroxymidazolam, and <13.8% for 4-hydroxymidazolam.

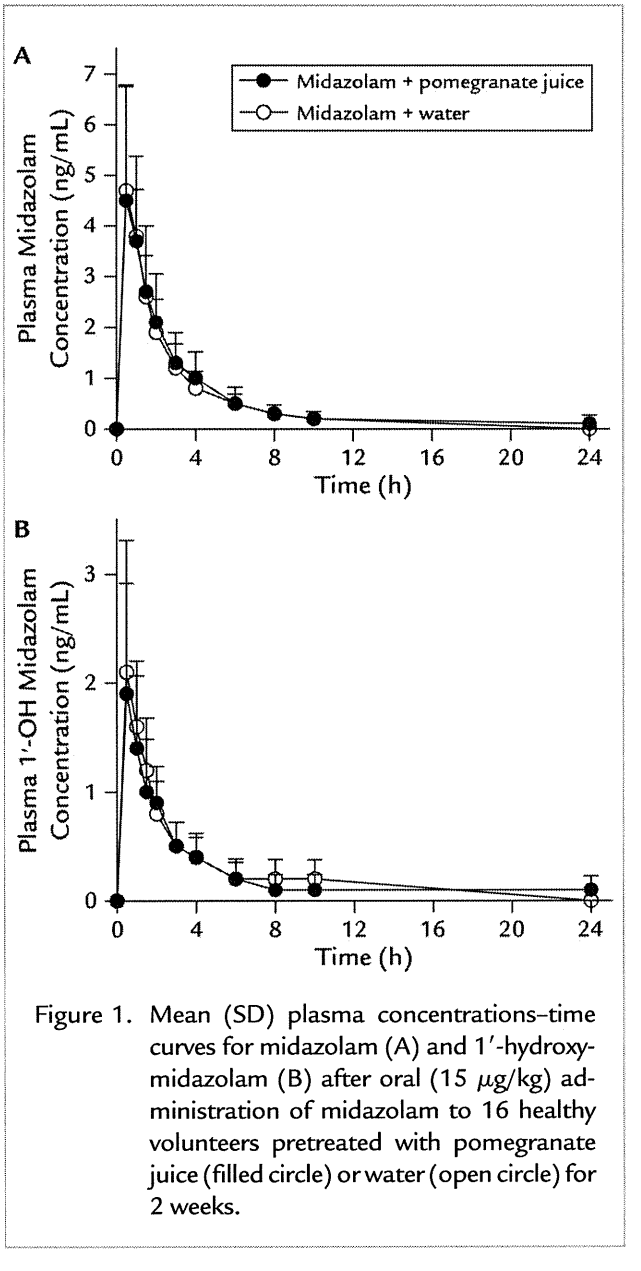

Figure 1. Mean (SD) plasma concentrations–time curves for midazolam (A) and 1′-hydroxymidazolam (B) after oral (15 μg/kg) administration of midazolam to 16 healthy volunteers pretreated with pomegranate juice (filled circle) or water (open circle) for 2 weeks.

*Trademark: Dormicum® (Astellas Pharma, Tokyo, Japan).

Pharmacokinetic Analysis

Pharmacokinetic parameters of midazolam and its metabolites for individual subjects were analyzed by noncompartmental pharmacokinetic methods using WinNonlin 5.1 (Pharsight, Mountain View, California).[25] The elimination half-life ($t_{1/2}$) during the log-linear terminal phase was calculated from the elimination rate constant (λ_z) determined by a linear regression analysis. AUC was calculated by the trapezoidal method for the observed values and subsequent extrapolation to infinity ($AUC_{0-\infty}$). Oral clearance (CL/F) was calculated as dose/$AUC_{0-\infty}$. The volume of distribution (Vd/F) was determined by multiplying CL/F by the mean residence time. The maximum plasma concentration (C_{max}) and the time at the maximum plasma concentration (T_{max}) were obtained by inspection. The metabolic ratio, defined as the ratio of the amount of metabolites (1′-hydroxymidazolam and 4-hydroxymidazolam) to that of midazolam in urine, was calculated using postdose 24-hour urine samples.

Statistical Analysis

The data are expressed as mean (SD), except for T_{max}, which is presented as median with range. Log-transformed C_{max}, $t_{1/2}$, $AUC_{0-\infty}$, CL/F, and V_d/F were analyzed by a paired *t*-test for the comparison between pomegranate juice and water (control) using GraphPad Prism 4.03 (GraphPad software, San Diego, California). Untransformed T_{max} was compared using the Wilcoxon test. Differences were regarded as statistically significant at $P < 0.05$. For all variables, except T_{max}, 95% confidence intervals (CI) were calculated on the geometric mean ratio (GMR) (midazolam + pomegranate/midazolam + water) of midazolam and its metabolite. When the 95% CI crossed 1.0, the results were not regarded as significant. Using previously reported pharmacokinetic data on midazolam in healthy volunteers,[26] the number of subjects was estimated to be sufficient to detect a 20% difference in the $AUC_{0-\infty}$ of midazolam between control and pomegranate juice phases, with a power of 80% (α-level 5%).

RESULTS

All of the subjects completed the study, with 1 subject complaining about the pain in relation to the placement of the catheter. Since the dosage of midazolam used in this study (15 μg/kg) was much lower than that in clinical settings, the pharmacologic effect of midazolam, such as sedation, was not observed in all participants. The consumption of pomegranate juice for 14 days had no detectable effect on volunteers' blood biochemical and laboratory test values.

Plasma concentrations of midazolam and its metabolites were monitored after oral administration of midazolam with or without pomegranate juice (**Figure 1**). After repeated consumption of pomegranate juice for 2

Table. Mean (SD) pharmacokinetic parameters of midazolam and 1′-hydroxymidazolam after oral (15 μg/kg) administration of midazolam pretreated with water or pomegranate juice for 2 weeks in 16 healthy volunteers. Data are mean (SD) unless otherwise specified.

	Water (Control)	Pomegranate Juice	Geometric Mean Ratio (95% CI)
Midazolam			
C_{max}, ng/mL	5.1 (1.7)	5.0 (2.0)	0.95 (0.79–1.11)
T_{max}, median (range), h	0.5 (0.5–1.5)	0.5 (0.5–3.0)	0.779*
$t_{1/2}$, h	3.6 (1.7)	3.8 (2.0)	1.05 (0.81–1.30)
$AUC_{0-\infty}$, ng/mL/h	12.7 (4.4)	14.2 (6.6)	1.02 (0.95–1.09)
CL/F, mL/min/kg	22.0 (7.6)	21.4 (10.2)	0.99 (0.97–1.01)
V_d/F, L/kg	6.2 (2.7)	6.5 (3.5)	1.00 (0.97–1.02)
1′-Hydroxymidazolam			
C_{max}, ng/mL	2.3 (1.1)	2.0 (0.8)	0.93 (0.72–1.15)
$AUC_{0-\infty}$, ng/mL/h	8.4 (6.2)	8.3 (6.5)	0.96 (0.76–1.16)

CL/F = oral clearance; V_d/F = volume of distribution.
*Between treatment *P* value.

weeks, no significant differences were observed in the pharmacokinetic parameters of midazolam and 1′-hydroxymidazolam (**Table**). Specifically, the $AUC_{0-\infty}$ GMR (95% CI) for midazolam and 1′-hydroxymidazolam were 1.02 (0.95–1.09) and 0.96 (0.76–1.16), respectively, suggesting that pomegranate juice did not alter the plasma pharmacokinetic profile of midazolam significantly. Likewise, the C_{max} GMRs (95% CI) for midazolam and 1′-hydroxymidazolam were 0.95 (0.79–1.11) and 0.93 (0.76–1.16), respectively. The GMRs (95% CI) of $t_{1/2}$, CL/F, and V_d/F for midazolam were 1.05 (0.81–1.30), 0.99 (0.97–1.01), and 1.00 (0.97–1.02), respectively. In addition, the consumption of pomegranate juice did not significantly change the T_{max} (P = 0.779) of midazolam. 4-Hydroxymidazolam was not detected in plasma samples at any time points.

The urinary excretion of 1′-hydroxymidazolam was not significantly different between those in the control and pomegranate juice groups (P = 0.34, **Figure 2A**). Also, no significant difference was found in the urinary excretion of 4-hydroxymidazolam (P = 0.32, **Figure 2B**). The GMRs (95% CI) for recovered 1′-hydroxymidazolam and 4-hydroxymidazolam in the urine, expressed as a percentage of the dose, were 1.08 (0.83–1.32) and 1.07 (0.93–1.21).

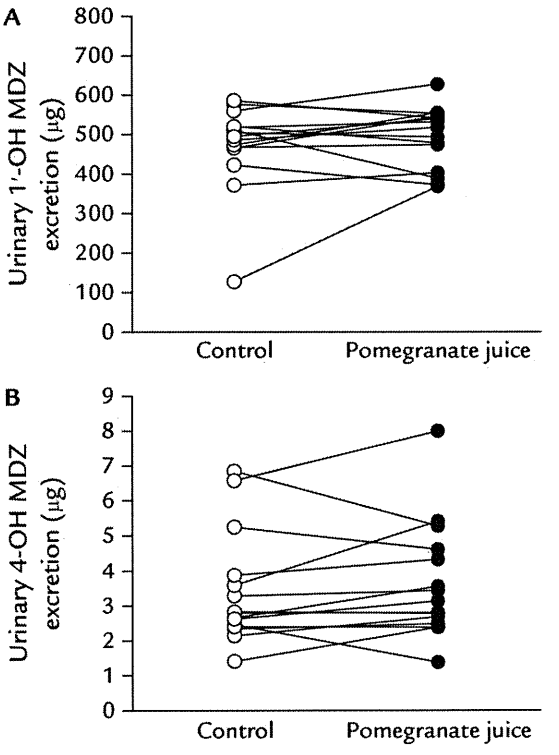

Figure 2. Individual values of urinary excretions of (A) 1′-hydroxymidazolam (1′-OH MDZ) and (B) 4-hydroxymidazolam (4-OH MDZ) after oral (15 μg/kg) administration of midazolam to 16 healthy volunteers.

DISCUSSION

Previously, the effect of a single 8-ounce glass of pomegranate juice on CYP3A activity had been examined in healthy volunteers.[22] It was also reported that multiple-dose administration of fruit juice exerted a different effect on drug metabolism than that of single administration.[27] At present, a literature search of MEDLINE and Cochrane databases has uncovered no clinical study investigating the long-term effect of pomegranate juice on the pharmacokinetics of CYP3A substrate. Hence, we attempted to elucidate whether the repeated consumption of pomegranate juice alters oral midazolam pharmacokinetics and found that after 2 weeks there was little difference in the plasma concentration profile of midazolam between the pomegranate juice phase and the control phase. This finding is supported by the previous report showing that the single coadministration of midazolam with pomegranate juice did not change either the oral or the systemic clearance of midazolam.[22] Therefore, repeated consumption of pomegranate juice may not cause clinically relevant interaction with midazolam in humans.

In this study, the pharmacokinetic parameters of midazolam, including AUC, C_{max}, T_{max}, $t_{1/2}$, and oral clearance, were not changed significantly in the pomegranate juice phase compared with the control phase. Also, there were no detectable differences in AUC and C_{max} of 1′-hydroxymidazolam between the pomegranate juice group and the control group. Although the urinary excretions of midazolam metabolites were not altered significantly between the control and pomegranate juice groups, individual subjects responded differentially to pomegranate juice. This raises the possibility that urinary excretions of midazolam metabolites may be affected by pomegranate juice in some patients. However, owing to the subset and small number of subjects, we did not confirm the influence of pomegranate juice on the urinary excretions of midazolam and its metabolites. The results of the present study contrast with those of an in vivo study in rats,

which reported that an oral administration of both pomegranate juice and grapefruit juice led to the marked decrease of the AUC of carbamazepine.[19] In addition, there are a few reports showing that pomegranate juice could inhibit triazolam α-hydroxylation and carbamazepine 10,11-epoxidation metabolism in human liver microsomes with similar potency to that of grapefruit juice.[19,22] One possible explanation for the inconsistent outcomes is the species differences in CYP enzymes between humans and rodents. In this context, Farkas et al discussed that CYP3A isoforms, susceptibility of enzymes to inhibitory compounds, and even pharmacokinetics of midazolam in humans were significantly different from those in rats.[22]

Variability of components of pomegranate juice could partially explain some of the differences between the results of the rat study and those of the clinical trial. Previous study suggested that the contents of potentially contributing compounds in grapefruit juice might crucially influence the magnitude of drug interaction and impair direct comparison of studies in which different juices have been used.[28] In the present and another clinical study, pomegranate juice was obtained from a commercial source.[22] In the rat study, on the other hand, pomegranate juice was freshly squeezed immediately before the experiment.[19] Since various ingredients originally present in pomegranate fruit may be lost or degraded during the manufacturing process and storage, it is possible that the components of marketed juices are different from those of freshly prepared pomegranate juice. Therefore, the effects of the consumption of fresh pomegranate fruit on the pharmacokinetics of CYP3A substrates may need to be addressed.

Nagata et al reported that pomegranate juice could inhibit human CYP2C9 activity and increase tolbutamide bioavailability in rats, suggesting that pomegranate juice could suppress CYP2C9 activity.[20] Two case reports also suggested the possible interaction between pomegranate juice and warfarin, a well-known CYP2C9 substrate.[18,29] With respect to the drug transporters, a recent report indicated that flavonoids such as quercetin efficiently inhibited OATP1A2 and OATP2B1 activities in vitro.[30] Thus, the inhibitory potency of pomegranate juice on the activity of CYP2C9, as well as drug transporters, should be investigated in clinical settings.

CONCLUSION

In this small, Japanese, adult volunteer population receiving a single subtherapeutic dose of midazolam, 2 weeks' consumption of pomegranate juice did not significantly alter the pharmacokinetic profile of midazolam compared with the results in the control group.

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ORIGINAL ARTICLE
Pulmonary Circulation

A Phase III, Multicenter, Collaborative, Open-Label Clinical Trial of Sildenafil in Japanese Patients With Pulmonary Arterial Hypertension

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Background: There is evidence that phosphodiesterase type-5 is effective for the treatment of pulmonary arterial hypertension (PAH).

Methods and Results: A phase III, multicenter, open-label clinical trial of sildenafil 20 mg t.i.d. was conducted in 21 Japanese patients with PAH to examine its efficacy, safety, and pharmacokinetics. The present trial consisted of a screening period and 12-week treatment. Patients who were enrolled in the present trial increased their 6-min walking distance of administration increased at week 12 by 84.2 m from baseline. Hemodynamic parameters (eg, mean pulmonary artery pressure and pulmonary vascular resistance), Borg dyspnea scores, and plasma brain natriuretic peptide concentrations also improved compared to baseline. Most patients improved or sustained WHO functional class. Seven subjects, who were examined for the pharmacokinetics of sildefanil, showed relatively large interindividual variations in the C_{max}, AUC_{0-8}, $C_{ss,av}$, and C_{trough} of the drug. Any serious adverse events, severe adverse events, and deaths were not observed. Most of events of undeniable causality were mild or moderate in severity. Sildefanil was well tolerated by the subjects.

Conclusions: Sildenafil 20 mg t.i.d. was effective and safe for Japanese patients with PAH. (*Circ J* 2011; **75:** 677–682)

Key Words: Efficacy; Pharmacokinetics; Pulmonary arterial hypertension; Safety; Sildenafil

Pulmonary arterial hypertension (PAH) is a group of pathologies with a poor prognosis that are featured by progressive obliteration of the small pulmonary vascular bed and a progressive increase in pulmonary vascular resistance (PVR), eventually leading to refractory right heart failure and premature death.[1–3] A national prospective registry in the United States[4] has reported that the estimated median survival of patients with primary pulmonary hypertension (PPH) who were untreated following a definite diagnosis was 2.8 years and that the estimated 5-year survival rate was 34%. PAH is diagnosed when a mean pulmonary artery pressure (mPAP) is greater than 25 mmHg at rest.[5] The disease is classified the following into several categories: PAH of unknown etiology (idiopathic or familiar) and PAH associated with collagen vascular disease, congenital systemic-to-pulmonary shunts, portal hypertension, human immunodeficiency virus (HIV) infections, drugs/toxins, and others.[6] PAH provokes exertional dyspnea, easy fatigability, palpitation, chest pain, syncope, cough, and/or other symptoms and considerably deteriorates quality of life of the patients.

In Japan, PPH is designated for listing in the Specified Disease Treatment Research Program, and in 2004 there were 758 patients with identifiable PPH.[7] The number of patients with PAH, including patients with PAH associated with

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underlying disorders (eg, collagen vascular disease and congenital heart diseases), is estimated to be approximately 6,000 in Japan. Therapeutic drugs for PAH have been used in recent years in addition to treatment with conventional agents (anticoagulants, diuretics, inotropic agents, supplemental oxygen, calcium-channel blockers, vasodilators, antiproliferative agents, and endothelin-receptor antagonists).[8]

New insights have been obtained on the pathogenesis of PAH thorough the action of various enzymes. For instance, Rho-kinase activation was recently found to be involved in the pathogenesis of PAH. The inhibition of Rho-kinase reduces monocrotaline-induced PAH, and the phosphorylation of RhoA and prevention of its translocation to the plasma membrane mediate hypoxia-induced PH. Furthermore, there is direct evidence for Rho-kinase activation in PAH patients.[9–11]

Phosphodiesterase type-5 (PDE-5) is strongly expressed in the lung, and PDE-5 gene expression and activity are increased in chronic PH.[12–14] Based on these findings, the inhibition of PDE-5 has been researched as a mechanism of a new approach to the treatment of PAH. Sildenafil (Viagra®), a potent and highly selective inhibitor of PDE-5 that metabolizes cGMP,[15–18] was approved as a therapeutic drug for male erectile dysfunction. cGMP-specific PDE-5 is abundantly present in the pulmonary vasculature, and sildenafil leads to nitric oxide-mediated vasodilation and decreases PVR.[19,20]

The present study was designed as a small-scale clinical trial to clarify the features of sildenafil as a therapeutic drug for PAH in Japan, and its objective was to examine the efficacy, safety, and pharmacokinetics of sildenafil administered orally to Japanese patients with PAH at the regimen of 20 mg t.i.d. for 12 weeks.

Methods

Between April 2007 and February 2009, the phase III, multicenter, collaborative, open-label clinical trial of sildenafil 20 mg t.i.d. was conducted in 21 Japanese patients with PAH who met all inclusion criteria, who did not fall under any exclusion criterion, and who were enrolled at 8 medical institutions in Japan. The protocol of the present trial was approved by the institutional review board or ethical review board at each institution, and the trial was conducted in accordance with the protocol. All subjects provided written informed consent before their enrollment.

The present clinical trial consisted of the screening period and 12-week treatment period. Throughout the treatment period, one 20-mg tablet of sildenafil citrate was orally administered t.i.d. to each subject at intervals ≥6 h. Subjects visited the hospital 5 times (visit 1 at screening, visit 2 at the onset of administration, visit 3 at week 4 of administration, visit 4 at week 8 of administration, and visit 5 at week 12 of administration or at discontinuation), together with contact by phone at week 1 of administration.

The objectives of the present trial were as follows: 1) to verify the efficacy and safety of sildenafil 20 mg t.i.d. administered orally to Japanese patients with PAH for 12 weeks; and 2) to examine the steady-state pharmacokinetics of sildefanil and its metabolite under these conditions of administration.

The main inclusion criteria were as follows: the male or female patient should be aged ≥16 years, should be diagnosed with PAH, and should have a mean PAP >25 mmHg and a pulmonary capillary wedge pressure (PCWP) <15 mmHg in right heart catheterization at screening or baseline.

Assessment of Efficacy

The primary endpoints for efficacy were as follows: 1) change in 6-min walking distance at week 12 of administration from baseline; and 2) changes in baseline hemodynamic parameters [mean PAP, PVR, and cardiac output (CO)] at week 12 of administration from baseline.

The secondary endpoints for efficacy were as follows: 1) change in baseline 6-min walking distance at week 8 of administration from baseline; 2) changes in baseline WHO functional class at weeks 4, 8, and 12 of administration from baseline; 3) changes in baseline hemodynamic parameters [PAP (systolic and diastolic), systemic blood pressures (systolic, diastolic, and mean), PCWP, right atrial pressure, cardiac index, heart rate, PVR index, systemic vascular resistance, systemic vascular resistance index, arterial oxygen saturation, arterial oxygen tension, mixed venous oxygen saturation, and mixed venous oxygen tension)] at week 12 of administration from baseline; 4) changes in baseline Borg dyspnea scores at weeks 8 and 12 of administration from baseline; and 5) changes in baseline plasma brain natriuretic peptide (BNP) concentration at weeks 4, 8, and 12 of administration from baseline.

Assessment of Pharmacokinetics

Subjects in the present trial were assessed for the plasma concentrations and pharmacokinetic parameters [time to reach maximum concentration (T_{max}), maximum concentration (C_{max}), and area under the curve (AUC_{0-8})] of sildefanil and its metabolite at steady state after sildefanil administration, and for the average plasma concentration at steady state ($C_{ss,av}$) and plasma through concentration (C_{trough}) at the steady state of sildefanil.

Blood for the pharmacokinetic assessment was collected before administration and at 0.5, 1, 1.5, 2, 4, 6, and 8 h after administration on the specified visit days. The pretreated samples of the blood collected were used to measure the plasma concentrations of sildefanil and its metabolite at Covance (Indianapolis, IN, USA) according to the liquid chromatography–tandem mass spectrometry method. The lower limit of quantification was 1.00 ng/ml for both sildenafil and its metabolite.

Assessment of Safety

Subjects in the present trial were assessed for adverse events during history taking, physical examinations, laboratory tests [hematology: hemoglobin, hematocrit, red blood cell count, platelet count, white blood cell count, differential white blood count (neutrophils, eosinophils, basophils, lymphocytes, and monocytes), and prothrombin time; and blood chemistry (total bilirubin, direct bilirubin, AST, ALT, ALP, γ-GTP, albumin, total protein, BUN, creatinine, sodium, potassium, uric acid, and BNP)], vital signs (blood pressure, pulse rate, and body weight), 12-lead electrocardiography, and ophthalmology (examination, visual acuity, color sense, and funduscopy).

Statistical Analysis

SAS software version 8.2 (SAS Institute Inc; Cary, NC, USA) was used to perform all statistical analyses according to Student's t-test. Full analysis set (FAS) was analyzed for efficacy, and FAS and per protocol set (PPS) were analyzed for primary endpoints. FAS consisted of subjects who received at least 1 dose of sildenafil and who were assessed for efficacy at baseline and after sildefanil administration. A P value of <0.05 was considered statistically significant.

PPS consisted of subjects in FAS who met the following

Table 1. Subject Disposition and Analysis Sets

	No. of subjects
Enrolled	21
Medicated	21
Completed	19
Discontinued	2
Analyzed for efficacy	
Full analysis set	20
Per protocol set	16
Assessed for pharmacokinetics	7
Assessed for safety	21
Adverse events	21
Laboratory tests	20

Table 2. Demographic Characteristics of Subjects and Their Features at Baseline

Background factors	No. of subjects
Gender	
Male	4
Female	17
Age (years)	
<18	0
18–44	9
45–64	10
≥65	2
Mean±SD	47.1±14.7
Minimum to maximum	19–68
Body weight (kg)	
Mean±SD	58.5±10.6
Minimum to maximum	38.1–84.0
WHO functional class	
I	0
II	7
III	14
IV	0

Table 3. Types of PAH and Duration of Disease

	No. of subjects
Idiopathic PAH	
No. of subjects	6
Duration of disease (years)	
Mean	1.46
Minimum to maximum	0.1–4.0
Familiar PAH	
No. of subjects	5
Duration of disease (years)	
Mean	1.15
Minimum to maximum	0.3–4.0
PAH associated with other disorders (eg, collagen vascular disease, congenital systemic to pulmonary shunts, portal hypertension, HIV infection, and drugs/toxins)	
No. of subjects	10
Duration of disease (years)	
Mean	3.33
Minimum to maximum	0.1–15.0

PAH, pulmonary arterial hypertension; HIV, human immunodeficiency virus.

Table 4. Combination Therapies (Therapeutic Drugs for PAH and Basic Therapeutic Drugs for PAH)

	No. of subjects
No. of subjects	21
Therapeutic drugs for PAH	
Beraprost	9
Basic therapeutic drugs for PAH	
Warfarin	9
Cardiotonic drugs (eg, digoxin)	0
Calcium-channel antagonists	12
Diuretics	21
Oxygen therapy	14

PAH, pulmonary arterial hypertension.

Table 5. Six-minute Walking Distance at Baseline and at Weeks 8 and 12 of Administration, as Well as Its Changes From Baseline in Subjects

Endpoint	Six-minute walking distance (m)	
	Actual value	Change from baseline
Subjects		
Baseline		
No. of assessed subjects	20	–
Mean±SD (95%CI)	326.0±86.2 (285.7, 366.3)	–
Week 8 of administration		
No. of assessed subjects	19	19
Mean±SD (95%CI)	410.2±72.9 (375.0, 445.3)	87.5±75.3* (51.2, 123.8)
Week 12 of administration or discontinuation (LOCF)		
No. of assessed subjects	20	20
Mean±SD (95%CI)	410.2±66.6 (379.0, 441.3)	84.2±74.9* (49.1, 119.2)

CI, confidence interval; LOCF, last observation carried forward.
*P<0.0001.

Table 6. Changes in Hemodynamic Parameters at Baseline and at Week 12 of Administration in Subjects

Hemodynamic parameter	Baseline (n=20)		12 week of administration (LOCF) (n=20)			
	Actual value		Actual value		Changes from baseline	
	Mean±SD	95%CI	Mean±SD	95%CI	Mean±SD	95%CI
Systolic pulmonary artery pressure (mmHg)	75.3±18.5	66.6, 84.0	72.0±20.9	62.2, 81.7	−3.4±13.4	−9.6, 2.9
Diastolic pulmonary artery pressure (mmHg)	30.1±12.4	24.2, 35.9	26.9±11.9	21.3, 32.5	−3.2±8.3	−7.0, 0.7
Systolic systemic arterial pressure (mmHg)	115.4±17.5	107.2, 123.5	116.1±16.1	108.5, 123.6	0.7±16.5	−7.0, 8.4
Diastolic systemic arterial pressure (mmHg)	68.3±14.8	61.3, 75.2	65.2±14.7	58.3, 72.1	−3.1±9.0	−7.3, 1.2
Mean systemic arterial pressure (mmHg)	88.5±19.0	79.6, 97.4	87.7±18.7	78.9, 96.4	−0.9±12.9	−6.9, 5.2
Pulmonary capillary wedge pressure (mmHg)	8.48±2.48	7.31, 9.64	9.15±3.15	7.68, 10.62	0.68±3.14	−0.79, 2.14
Right atrial pressure (mmHg)	6.6±3.4	5.0, 8.2	6.4±3.6	4.6, 8.1	−0.3±4.4	−2.3, 1.8
Cardiac index ($L \cdot min^{-1} \cdot m^{-2}$)	2.35±0.78	1.98, 2.71	2.67±0.99	2.20, 3.13	0.32±0.62	0.03, 0.61
Heart rate (beats/min)	73.59±15.05	66.54, 80.63	69.45±15.98	61.97, 76.93	−4.14±7.45	−7.62, −0.65
Pulmonary resistance index (dyne·s/cm^5/m^2)	1,581.31±791.94	1,210.67, 1,951.95	1,199.31±660.73	890.09, 1,508.54	−382.00±491.80	−612.17, −151.83
Systemic vascular resistance (dyne·s/cm^5)	1954.86±945.04	1,512.57, 2,397.16	1,689.09±606.04	1,405.45, 1,972.73	−265.77±785.52	−633.41, 101.86
Systemic vascular resistance index (dyne·s/cm^5/m^2)	3,127.11±1,564.66	2,394.82, 3,859.39	2,717.22±1,027.45	2,236.35, 3,198.08	−409.89±1271.30	−1,004.88, 185.09
Mixed blood oxygen saturation index (%)	65.37±9.74	60.81, 69.93	68.28±5.82	65.56, 71.00	2.91±9.05	−1.33, 7.15
Atrial blood oxygen saturation (%)	92.930±6.877	89.711, 96.149	93.370±3.799	91.592, 95.148	0.440±5.437	−2.104, 2.984
Atrial oxygen tension (mmHg)	74.36±15.63	67.05, 81.67	72.35±12.09	66.69, 78.00	−2.02±11.17	−7.24, 3.21
Mixed venous oxygen tension (mmHg)	36.55±4.33	34.46, 38.64	37.12±2.67*	35.83, 38.40*	0.57±4.35*	−1.53, 2.66*

*n=19.
n, no. of evaluated patients; LOCF, last observation carried forward; SD, standard deviation; CI, confidence interval.

criteria: primary endpoints are assessed; there is no violation of the inclusion and exclusion criteria that could possibly affect primary endpoints; any combination-prohibited drug with possible effects on primary endpoints is not used during the study period; and the medication adherence 80–100%. Summary statistics and 95% confidence intervals for the means were calculated with respect to the actual values of the 6-min walking distance, hemodynamic parameters, Borg dyspnea score, and plasma BNP concentration and to their changes from baseline.

The WinNonlin software version 4.1 (Pharsight Corp.; Mountain View, CA, USA) was used to determine pharmacokinetic parameters of sildenafil and its metabolite according to the noncompartment model method in subjects who did not receive any other therapeutic drug for PAH, who met all inclusion criteria for pharmacokinetic assessment and who did not fall under any exclusion criteria for the assessment. All subjects who received at least 1 dose of sildenafil were assessed for safety.

Results

Subject disposition and analysis sets are shown in **Table 1**. Twenty-one subjects were enrolled, 2 of whom discontinued the trial. Therefore, 19 subjects completed the trial. Of those who discontinued, 1 showed insufficient efficacy and 1 had an adverse event.

Among 21 subjects who were enrolled, 1 and 5 were excluded from FAS and PPS, respectively. The former patient was excluded due to no postdose measurement of all endpoints for efficacy. The latter patients were excluded from PPS because of the following reasons: 2 subjects violated the inclusion/exclusion criteria; 1 subject was excluded from FAS; 1 subject was not evaluated for primary endpoints; and 1 subject ingested a combination-prohibited drug.

The demographic characteristics of subjects and their features at baseline are shown in **Table 2**. The percentages of males and females were as follows: 19.0% (4 males) and 81.0% (17 females). Subjects had different baseline WHO functional classes: 7 with class II and 14 with class III.

The types of PAH and duration of disease are shown in **Table 3**. Among the subjects, 6, 5, 10 were diagnosed with idiopathic PAH, familiar PAH, and PAH associated with underlying disorders, respectively.

Therapeutic drugs for PAH and basic therapeutic drugs for PAH, which were administered in combination with sildenafil during the study period, are shown in **Table 4**. The major therapeutic drug for PAH, which was used in combination therapies during the study period, was beraprost, and the major basic therapeutic drugs for PAH were diuretics and oxygen therapy. Among the subjects, 9 received beraprost in combination with sildenafil; 12 received sildenafil alone.

Efficacy

The actual values of 6-min walking distance at baseline and at weeks 8 and 12 of administration, as well as changes in 6-min walking distance from baseline are shown in **Table 5**. At week 8 of administration, 6-min walking distance improved statistically significantly ($P<0.0001$) by 87.5 m from baseline. Therefore, the distance was shown to have improved as much at week 8 of administration as at week 12 of administration. At week 12 of administration, the 6-min walking distance had also improved statistically significantly ($P<0.0001$) by 84.2 m from baseline.

The actual values of hemodynamic parameters (mean PAP,