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Pharmacokinetic Characteristics of Levosulpiride in Relation to the Genetic Polymorphism of *MDR1*: From Knockout Mouse to Human

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ABSTRACT – The purposes of this study were to clarify the involvement of P-glycoprotein (P-gp) in the efflux of levosulpiride in knockout mice that lack the *mdr1a1b* gene and to evaluate the relationship between the genetic polymorphisms in *MDR1* gene (exon 21) and levosulpiride disposition in healthy Korean subjects. After oral administration (10 μg/g) of levosulpiride to *mdr1a/1b(-/-)* and wild-type mice, plasma and brain samples were obtained at 45 min. We also investigated the genotype for *MDR1* (exon 21) gene in humans using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. A single oral dose of 25 mg levosulpiride was administered to 58 healthy subjects, who were based on the *MDR1* genotype for the G2677T SNP. Blood samples were taken up to 36 hr after dosing. The concentrations of levosulpiride in mouse plasma and brain were statistically significant difference between the two animal groups ($P<0.05$). In addition, the average brain-to-plasma concentration ratio (*Kp*) of levosulpiride was 3.4-fold ($P<0.01$) higher in the *mdr1a/1b(-/-)* mice compared with the wild-type mice. We also found that the values of $AUC_{0-\infty}$, partial AUC (AUC_{0-4h}) and C_{max} were significantly different between homozygous 2677TT subjects and the subjects with at least one wild-type allele (GG and GT subjects, $P=0.012$ for $AUC_{0-\infty}$; $P=0.008$ for AUC_{0-4h}; $P=0.038$ for C_{max}). The results confirm that levosulpiride is a P-gp substrate *in vivo*, and clearly demonstrate the effect of SNP 2677G>T in exon 21 of the *MDR1* gene on levosulpiride disposition.

Key words – Levosulpiride, P-glycoprotein, *MDR1*-knockout mouse, Genetic polymorphism, Pharmacokinetics

Levosulpiride, the levorotatory enantiomer of sulpiride, is an oral selective dopamine D_2 receptor antagonist and is widely used for the treatment of schizophrenia, depression, other related psychological disorders, and gastric and duodenal ulcers.[1,2] Optimization of treatment with levosulpiride and maintaining an effective blood concentration require knowledge of its bioavailability, pharmacokinetics, and pharmacodynamics in humans. Levosulpiride has a peptide bond and two ionizable species, the nitrogen of the pyrrolidine ring (pK_{a1} 8.99) and the sulphamoyl group (pK_{a2} 10.19) in its structure (Figure 1). Mizuno et al.[3] showed that the sulpiride is mainly absorbed from the intestine and excreted by the kidney, mostly as an unchanged compound after oral administration. The bioavailability of sulpiride in human, rat and dogs are approximately 27, 15~20, and 44~85%, respectively.[3-5] The low bioavailability in human is probably due to incomplete absorption.[4]

Recently, Watanabe et al.[6,7] demonstrated that secretion of sulpiride across human intestinal Caco-2 cells was mediated by the transport systems of P-glycoprotein (P-gp) and organic cation. Baluom et al.[8] also reported that, in the rat model used, bioavailability of sulpiride was increased when co-administered with verapamil, and quinidine, P-gp inhibitors. Therefore, their results could be one of the reasons why the poor oral bioavailability of sulpiride in human after oral administration was caused by P-gp efflux on the brush-border membrane.

P-gp, a product of the multidrug resistance gene (*MDR1*), is a member of the adenosine triphosphatase-binding cassette (ABC) superfamily of transporters.[9] P-gp was initially identified in tumor cells where its over-expression was found to be a cause of resistance to multiple chemotherapeutic agents.[10] P-gp has also been found to mediate the energy-dependent efflux of xenobiotics in normal tissues, including the gastrointestinal mucosa, liver canalicular membrane, and kidney proximal tubules, as well as blood-brain barriers (BBB) such as the brain and placenta.[11-17]

Mammalian P-gps are encoded by a family of closely related genes, including two in humans (*MDR1* and *MDR3*) and three in mice (*mdr1a, mdr1b* and *mdr2*).[13,18-20] The human *MDR1* and mouse *mdr1a* and *mdr1b* P-gps can confer MDR and are referred to as drug-transporting or mdr1-type P–gps. Knockout mice in which either the *mdr1a* gene or both the *mdr1a* and *1b*

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genes are disrupted have been especially useful in determining the importance of P-gp in a particular drug's disposition, since in these animals the transporter is essentially or completely absent, respectively. These experimental data obtained using knockout mice provide strong evidence that the general observations of a wide variation in response to certain drugs among different-individuals may have a genetic basis. In the last several years, single nucleotide polymorphisms (SNPs) within *MDR1* gene have been identified and characterized that could potentially affect the pharmacokinetics and pharmacodynamics of drugs that are P-gp substrates.[21-24] Two SNPs in particular, C3435T in exon 26 and G2677T in exon 21, have been examined in detail and found to be associated with different changes in plasma drug concentration and phenotypic effects.

It has already been shown that levosulpiride is a substrate of P-glycoprotein, by means of *in vitro* studies using Caco-2 cells. In addition, the disposition of levosulpiride, is complicated by the fact that levosulpiride disposition involves many other transporters including OCT, PEPT1, and OCTN.[6,7] However, the involvement of P-glycoprotein in the *in vivo* tissue-distribution, absorption, and elimination of levosulpiride has not yet been clarified.

Therefore, the aim of this study was to characterize the role of P-gp in the disposition of levosulpiride using the *mdr1a/1b* knockout mouse model, which has to our knowledge not been previously applied to any sulpiride. Finally, the relationship between the *MDR1* polymorphism (the G2677T SNP in exon 21) and levosulpiride disposition in a genotype group of healthy Korean subjects was investigated.

Materials and Methods

Materials

Levosulpiride (99.77%) was provided by the Research Institute of Kuhnil Pharmaceuticals (Seoul, Korea). Tiapride hydrochloride (I.S.) was purchased from Sigma Co. (St. Louis, MO, USA). Normal saline (Choongwae Pharma Co., Seoul, Korea) and heparin sodium (25,000 IU/mL, Green Cross, Seoul, Korea), diethyl ether (Dae-Jung, Incheon, Korea) were used for animal experiments.

Methanol and acetonitrile (HPLC grade) were purchased from Fischer Scientific (Fair Lawn, NJ, USA) and dichloromethane, potassium dihydrogenate phosphate (Yakuri Pure Chemical Co., Ltd., Osaka, Japan), sodium borate buffer (pH 8.5, Sigma Co., St. Louis, MO, USA) were used for HPLC assay. The other chemicals were of HPLC grade or highest quality available. HPLC grade water was obtained from a Milli-Q water purification system (Millipore Co., Milford, MA, USA) and used throughout the study. The mobile phase components such as acetonitrile, potassium dihydrogenate phosphate were filtered through a 0.45-μm pore size membrane filter prior to mixing and ultrasonically degassed after mixing.

Animals

Male *mdr1a/1b*(-/-) transgenic mice (FVB/NTacFBR-[KO]*mdr1a*-[KO]*mdr1b* N7) and genetically matched male wild-type *mdr1a/1b*(+/+) mice (FVB/NTacFBR) aged 8 weeks weighing 20-26 g were purchased from Taconic Farms Inc. (Germantown, NY, USA). These mice were originally created by sequential gene targeting in 129/Ola E14 embryonic stem cells, and back-crossed seven times (N7) to FVB/N from the C57BL/6 × 129 chimera. These mice, developed by Schinkel et al.,[15] are functionally deficient in the blood-brain barrier and have shown to accumulate much higher amounts of certain drugs in their brain. The genetic authenticity of each parent of these mice was verified by polymerase chain reaction (PCR) on genomic DNA to be *mdr1a/1b*. The mice were housed individually and maintained on a 12 hr/12 hr light/dark cycle at 23 ± 1°C and 50% relative humidity, and acclimated in the College of Pharmacy at CNU Animal Resources facility for 2 weeks with food and tap water *ad libitum*. Before the experiment, mice were fasted overnight but given free access to water.

The mice of each group (n=6), FVB and MDRK, were randomly subsequently tagged. Levosulpiride dissolved in 0.5% weak acetic acid. A dose of 10 μg/g was orally administered in an entire volume of 10 μL/g mouse. Each group mouse was subjected to only one blood sampling by inferior vena cava in heparinized syringe under light ether anesthesia at 45 min. The selected time point (45 min) and dose (10 μg/g mouse) were determined as around T_{max} (time to reach the peak plasma concentration) and the measurable administered dose in the brain through pre-experiment for wild-type mice, respectively. Trunk blood (about 1 mL) was collected in EDTA-coated tubes (K3 EDTA Vacutainer®, 13 × 15 mm, Becton Dickinson, Maylan, UK) and centrifuged at 3000 g for 2 min for the determination of plasma concentration of levosulpiride. After collection of blood sample, brain was immediately dissected, washed with saline, blotted on paper towels, and weighted and then homogenized in ten-fold volume of a sodium borate buffer (pH 8.5). The plasma and brain homogenates were kept at − 80°C until assay.

Levosulpiride disposition in healthy subjects

Studies were undertaken in 58 healthy male Korean subjects

who were based on the G2677T *MDR1* genotype, in 20-32 years old, weighing 50 to 84 kg. *MDR1* G2677T genotype was determined by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP).

On the basis of medical history, physical examination, and routine biochemical laboratory test results, all of the subjects were considered healthy. During 7 days before the study, subjects did not take any drugs and did not drink any alcohol. On the study day, each subject received a 25-mg oral dose of levosulpiride, after an overnight fast. Blood samples (8 mL) were obtained through an indwelling cannula at 0, 1, 2, 3, 4, 6, 8, 12, 24, and 36 hr after drug administration. Informed consent was obtained from the subjects after explaining the nature and purpose details of the study. The study protocol was approved by the Institutional Review Board (IRB) of Institute of Bioequivalence and Bridging Study, Chonnam National University, Gwangju, Korea. This study was performed according to the revised Declaration of Helsinki for biomedical research involving human subjects and the rules of Good Clinical Practice.

HPLC analysis of levosulpiride concentrations in plasma and brain

Plasma and brain samples were extracted and determined using an HPLC method previously developed and validated in our laboratory.[25] Briefly, 20 μL or 100 μL of internal standard working solution (tiapride 1 μg/mL), 50 μL or 200 μL of 0.1 M sodium hydroxide, and 50 μL or 200 μL of 0.1 M sodium borate (pH 8.5) were added to 200 μL or 1 mL of plasma or brain samples, and mixed by vortexing for 3 sec. The mixture was extracted with 1 or 4 mL of dichloromethane by vortex-mixing for 2 min and centrifuged at 3,000 *g* for 10 min. The aqueous layer (upper phase) was discarded by aspiration with a Pasteur pipette and organic layer (lower phase) was transferred to another clean tube. The organic layer was back-extracted with 200 μL of 0.025 M sulfuric acid by vortex-mixing for 1 min and centrifuged at 3,000 *g* for 5 min. Subsequently, a 150 μL aliquot of the supernatants was transferred into a clean Eppendorf tube and 20 μL of 0.5 M sodium hydroxide was added. A 50 μL of the solution was injected directly onto the HPLC system. The HPLC system consisted of a model LC-10AD isocratic pump (Shimadzu, Kyoto, Japan), equipped with a Rheodyne 7725 injection valve (Rheodyne, Cotati, CA, USA) and a model RF-10A_{XL} fluorescence detector (Shimadzu, Kyoto, Japan). Levosulpiride and internal standard were separated by a LUNA C_{18}(2) column (5-μm particle size, 250× 4.6 mm I.D.; Phenomenex, Torrance, CA, USA) with security guard cartridge (KJ0-4282, 5-μm particle size, 4 × 2.0 mm I.D.; Phenomenex, Torrance, CA, USA) using a mixture of acetonitrile–0.01 M potassium dihydrogen phosphate (30:70, v/v, adjusted to pH 8.5 with triethylamine) at a flow rate of 0.8 mL/min with a column inlet pressure of about 9 MPa. The column was operated at 30°C in column oven (ThermaSphere™ TS-130, Phenomenex, Torrance, CA, USA), and the effluent was monitored at an excitation wavelength of 300 nm and emission wavelength of 365 nm. Detector output was quantitated on a model Class LC-10 integrator (Shimadzu, Kyoto, Japan).

Quantitation of levosulpiride was determined by weighted linear regression analysis of peak area ratio versus concentrations of added levosulpiride employing 1/concentration as the weighting factor. The calibration curves were linear over the respective concentration range. The intra-day precision of the assay was assessed by calculating the coefficients of variation (C.V.) for the analysis of QC samples in five replicates, and inter-day precision was determined through the analysis of QC samples on five consecutive days. Accuracy was determined by comparing the calculated concentration using calibration curves to known concentration. The lower limit of quantitation (LLOQ) was defined as the lowest concentration 10 times of S/N ratio yielding a precision less than 20% (C.V.) and accuracy between 80 and 120% of the theoretical value. The absolute recoveries from human plasma were assessed by comparison of the peak area from extracted QC samples to the area of standard corresponding to respective concentrations followed by application of a correction factor.

Under these conditions, the internal standard and levosulpiride eluted at 7.0 and 5.5 min, respectively. Calibration curves were constructed between 0.25-200 ng/mL and the LLOQ was 0.25 ng/mL for human plasma. Within-day coefficients of variation in quality control samples (n=5 for each) of human plasma at low (25 ng/mL), medium (100 ng/mL), and high (200 ng/mL) concentrations were 6.2, 4.4, and 4.1%, respectively. Inter-day coefficients of variation of the assay were below 15% for human plasma levosulpiride. Mouse plasma and brain samples were calibrated ranging from 2 to 1000 ng/mL for mouse plasma and 0.5 to 100 ng/g for mouse brain samples. The concentrations were in the measurement range to the respective levosulpiride. The LLOQ of levosulpiride for mouse plasma and brain was 2 and 0.5 ng/mL, respectively. All the intra-day and inter-day coefficients of variation were lower than 7% at relevant concentrations (n=5). The plasma and brain extraction recoveries ranged from 85 to 98%.

Pharmacokinetic analysis

Pharmacokinetic parameters were calculated by non-com-

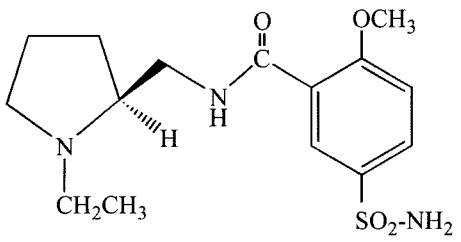

Figure 1–Chemical structure of levosulpiride.

partmental analysis using WinNonlin program (Ver.2.1, Pharsight Corporation, Mountain View, CA, USA). C_{max} and T_{max} were determined by the inspection from individual plasma concentration-time profiles. The area under the concentration-time curve from time zero to time infinity ($AUC_{0-\infty}$) was calculated as $AUC_{0-t}+C_t/\lambda_z$, where C_t is the last measurable concentration, λ_z is a terminal rate constant. AUC_{0-t} was calculated by the linear trapezoidal rule from 0 to 36 hs. The partial AUC (AUC_{0-4h}) representing the absorption phase of levosulpiride was also calculated by the linear trapezoidal rule from 0 to 4 hs.

Kp (brain-to-plasma concentration ratio) value was defined as following.

$$Kp = \frac{Brain\ concentration\ of\ levosulpiride\ (C_b)}{Pasma\ concentration\ of\ levosulpiride\ (C_p)}$$

Therefore, this value was used as evaluation factor affected by P-gp mediated transport. Extent of brain uptake was obtained by dividing the concentration of levosulpiride in the brain to that in plasma.

Statistical analysis

Data are shown as mean±S.D.. The differences between *mdr1a/1b*(-/-) and wild-type mice were tested for significance with the Mann-Whitney rank sum correlation test in the presence of nonparametric data distribution. A two-tailed *P* value was used and the level of statistical significance was set as $P<0.05$.

The effect of *MDR1* SNP G2677T on levosulpiride pharmscokinetics was first analyzed by the Kruskal-Wallis test. For each SNP, the groups were compared by the Mann-Whitney U test only if the global Kruskal-Wallis test was statistically significant ($P<0.05$).

Results

***Kp* value of levosulpiride in *mdr1a/1b*(-/-) and wild-type mice**

The concentrations of levosulpiride in mouse plasma and brain after oral administration of levosulpiride (10 μg/g) to *mdr1a/1b* knockout and wild-type mice were statistically significant difference between the two animal groups (Table I, $P<0.05$). In addition, the average brain-to-plasma concentration ratio (*Kp*) of levosulpiride was 3.4-fold ($P<0.01$) higher in the *mdr1a/1b(-/-)* mice compared with the wild-type mice (Table I).

Table I–*Plasma Concentrations and Brain-to-plasma Concentration (Kp) Value of levosulpiride in Mdr1a/1b(+/+) and Mdr1a/1b(-/-) Mice at 45 min after Oral Administration of 10 μg/g Levosulpiride (n = 6)*

Parameter	*mdr1a/1b(+/+)* mice	*mdr1a/1b(-/-)* mice	Ratio
Concentrations			
–*Plasma (ng/mL)* *	146.8±31.9	722.4±123.1	4.92
–*Brain (ng/g)* *	2.0±1.9	35.2±16.8	17.6
Brain / Plasma ratio			
–*Kp value***	0.013±0.007	0.044±0.007	3.38

*$P<0.05$ and **$P<0.01$, significant differences between the two groups.

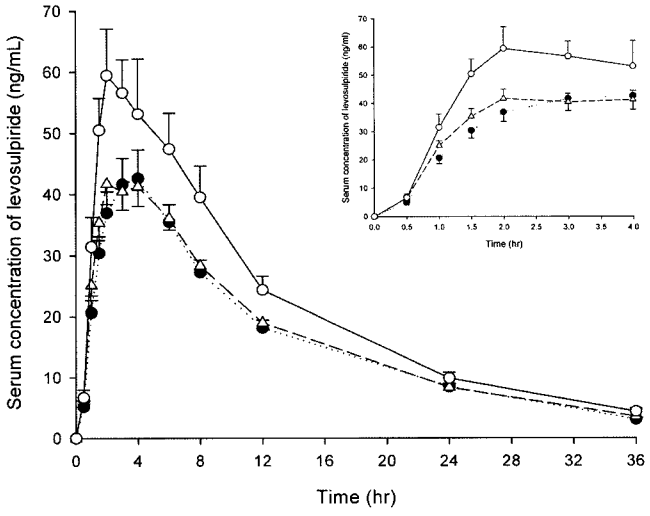

Figure 2–Mean plasma concentration-time profiles after oral administration of 25-mg levosulpiride in MDR1 G2677T in exon 21 genotype groups.

Effect of genetic polymorphism on levosulpiride disposition in human

G2677T *MDR1* genotyping revealed that 23 subjects (39.7 %) were GG, 28 GT (48.3%), and 7 TT (12.1%) (Table II). Mean plasma concentration time curves of the subjects stratified for genotypes at position 2677 (GG, GT and TT) are shown in Figure 2, and the corresponding PK parameters are shown in Table III. We found that the homozygous 2677TT subjects had significantly higher $AUC_{0-\infty}$ (GG and GT versus TT, 625.0±194.3 and 650.0±157.3 versus 837.9±193.6

Table II–*Characteristics of Healthy Male Subjects Grouped by Genotypes in Exon 21 (G2677T)*

MDR1 genotype	Subjects (No.)	Age (years)	Weight (kg)	Height (cm)	sGOT (U/L)	sGPT (U/L)	CL_{CR} (mL/min)
G2677T in exon 21							
GG	23	23.52 (2.502)	68.33 (11.19)	173.70 (6.167)	19.22 (5.526)	18.83 (7.802)	114.5 (13.72)
GT	28	23.71 (2.209)	67.41 (9.040)	173.4 (4.145)	17.71 (4.545)	18.00 (7.727)	110.3 (13.35)
TT	7	22.71 (1.799)	59.79 (7.491)	170.4 (2.330)	17.00 (2.380)	14.57 (5.593)	112.7 (15.76)

Data are given as mean (S.D.).

Table III–*Pharmacokinetic Parameters for Levosulpiride after Oral Administration (25-mg) in MDR1 Genotype of Healthy Korean Subjects*

Genotype	$AUC_{0-\infty}$ *(ng·hr/mL)*	AUC_{0-4} *(ng·hr/mL)*	C_{max} (ng/mL)	T_{max} (hr)	$t_{1/2}$ (hr)	CL/F (mL/min)
G2677T in exon 21						
GG (n=23)	625.0 (194.3)	118.7 (48.58)	50.39 (20.56)	3.717 (1.622)	9.617 (2.190)	728.7 (215.5)
GT (n=28)	650.0 (157.3)	125.8 (40.94)	52.36 (16.04)	3.054 (1.517)	9.727 (2.234)	677.5 (162.1)
TT (n=7)	837.9 (193.6)	172.0 (44.72)	68.72 (23.20)	3.143 (1.464)	9.629 (1.755)	518.5 (110.4)
P value	0.012	0.008	0.038	NS	NS	0.012

Data are given as mean (S.D.).
AUC_{0-4}, area under concentration-time curve from time 0 to 4 hs; C_{max}, peak plasma concentration; T_{max}, time to reach peak plasma concentration; $t_{1/2}$, half-life; CL/F, total clearance; NS, not significant.
*$P<0.05$, significant differences between carriers of two variant alleles (subjects with TT in G2677T) and carriers of at least one wild-type allele by Mann-Whitney *U* test.

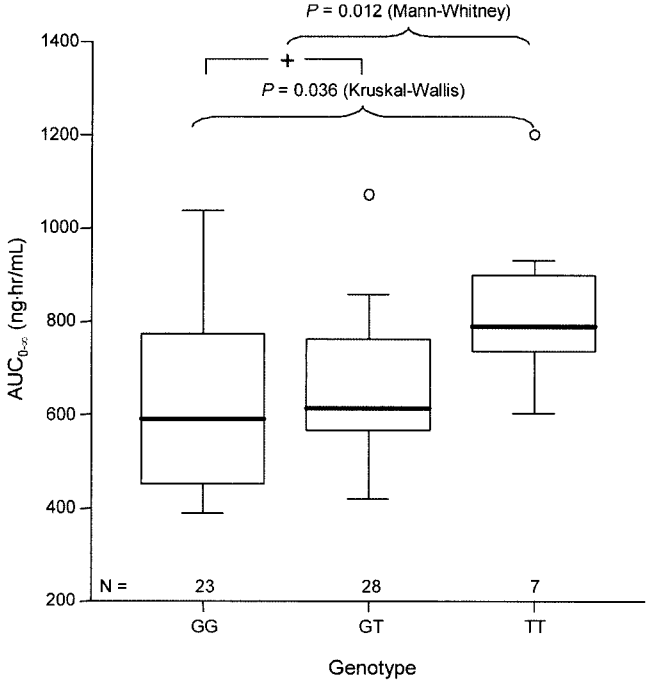

Figure 3–Effect of *MDR1* genotype G2677T in exon 21 on levosulpiride $AUC_{0-\infty}$. Correlation between the genotypes and $AUC_{0-\infty}$ was tested by Kruskal-Wallis test. Differences between carriers of two variant alleles (subjects with TT in case of G2677T) and carriers of at least one wild-type allele were evaluated with the Mann-Whitney *U* test.

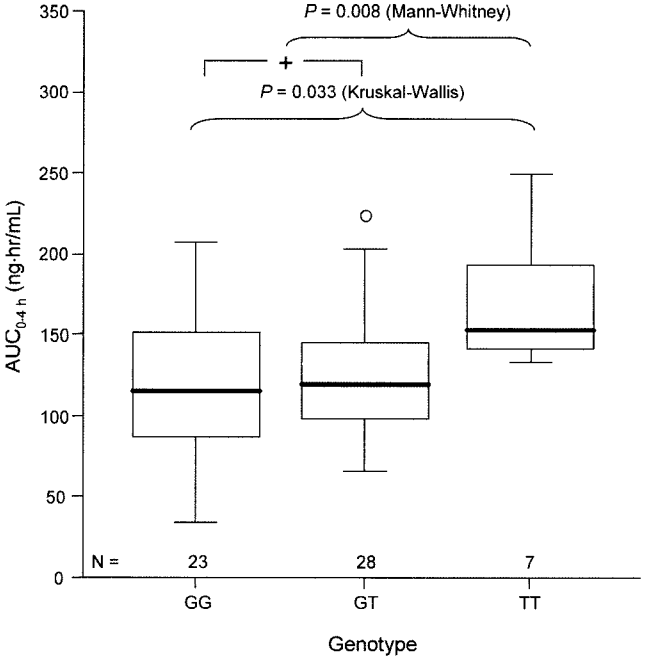

Figure 4–Effect of *MDR1* genotype G2677T in exon 21 on levosulpiride $AUC_{0-4\,h}$. Correlation between the genotypes and AUC was tested by Kruskal-Wallis test. Differences between carriers of two variant alleles (subjects with TT in case of G2677T) and carriers of at least one wild-type allele were evaluated with the Mann-Whitney *U* test.

ng · hr/mL; $P=0.012$). In case of $AUC_{0\text{-}4\,h}$, the homozygous TT subjects had significantly higher than the subjects with at least one wild-type allele (subjects with GG and GT, $P=0.008$), respectively (Figure 3 and 4).

Discussion

In the present study, we used *mdr1a/1b* gene-deficient mice to examine the influence of P-gp encoded by this gene on the *in vivo* disposition kinetics of levosulpiride. These results showed that there were significant differences in brain and plasma concentrations of levosulpiride at 45 min after oral administration of levosulpiride (10 μg/g) to wild-type and *mdr1a/1b*(-/-) mice (Table I). The *Kp* value of levosulpiride was 3.4 times higher in the *mdr1a/1b(-/-)* mice compared with that in the wild-type mice (Table I). Thus, it might be concluded that levosulpiride is also a P-gp substrate *in vivo* as suggested *in vitro*.[6,7]

The $AUC_{0\text{-}\infty}$, $AUC_{0\text{-}4\,h}$, and C_{max} values obtained following a single oral dose of levosulpiride were found to be significantly different between carriers of two variant alleles and of at least one wild-type allele of G2677T in exon 21 ($P=0.012$, $P=0.008$, and $P=0.038$, respectively). These results clearly demonstrated the effect of SNP 2677G>T in exon 21 of the *MDR1* gene on the pharmacokinetics of orally administered levosulpiride. Interestingly, this is the first study reporting the effect of the 2677T allele on the pharmacokinetics of levosulpiride.

This study also showed that genetic polymorphisms of the *MDR1* gene can have impact on levosulpiride disposition in human, and that these genetic differences can explain the intersubject variabilities of levosulpiride disposition in human.

Such knowledge of the genotype-phenotype correlation and frequency distribution of functional SNPs may be a valuable tool for individualizing drug therapy. This information can also be useful for explaining interindividual and inter-ethnic variations in drug response and/or its side effects.

Conclusion

In the present study, we used *mdr1a/1b* gene-deficient mice to examine the influence of P-gp encoded by this gene on the *in vivo* disposition kinetics of levosulpiride and tested the effect of SNP 2677G>T in exon 21 of the *MDR1* gene on levosulpiride disposition in humans. The results confirm that levosulpiride is a P-gp substrate *in vivo*, and clearly demonstrate the effect of SNP 2677G>T in exon 21 of the *MDR1* gene on levosulpiride disposition.

Acknowledgement

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