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Title page

Title: UDP-glucuronosyltransferase1A4 polymorphisms in a Japanese population and kinetics of clozapine glucuronidation.

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Running title page

Running title: UGT1A4 polymorphisms and their clozapine kinetics.

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Abstract

The UDP-glucuronosyltransferase (UGT) family plays a major role in the excretion of endobiotics and xenobiotics and their metabolites. Human UGT1A4 catalyzes the glucuronidation of primary, secondary and tertiary amines, sapogenins, androgens, and progestins. We directly sequenced PCR–amplified fragments of the *UGT1A4* gene from 100 healthy adult Japanese volunteers, and calculated their mutation frequency. We identified four single nucleotide polymorphisms (SNPs): three in exon 1 (142T>G: L48V, 448T>C: L150L, 804G>A: P268P), and one in intron 1 (867+43C>T). We found three types of alleles with distinct SNP combinations, that coded for different amino acid sequences: L48V-L150L-P268P-867+43C>T (frequency, 0.155), L48V (0.01), and P268P (0.01) (wild type frequency was 0.825). The L48V mutant gave twice the efficiency (*V*max/*K*m) for the antipsychotic drug clozapine than the wild type. Efficiencies of L48V for trans-androsterone, imipramine and cyproheptadine were increased, but the efficiency for tigogenin was reduced. L48V therefore increased or decreased the glucuronidation activity depending upon the substrates. This study shows the importance of identifying patients

with the L48V polymorphism when calculating dosage, and when considering the potential adverse effects of drugs that are substrates of UGT1A4.

Introduction

The UDP-glucuronosyltransferase (UGT) superfamily plays a major role in the excretion of endobiotics and xenobiotics and their metabolites (Miners and Mackenzie, 1991). Its members have widely overlapping substrate specificity and characteristic individual tissue distribution. UGT comprises two sub-families, UGT1 and UGT2. *UGT1* has a unique gene structure; there are thirteen exon 1s from *UGT1A1* to *UGT1A13P*, and exon 2 to 5 are used in common for all mRNAs expressed from the gene. Nine of these exon code for UGT1 isoforms (*UGT1A1, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9,* and *UGT1A10*) (Mackenzie et al, 1997) and four code for pseudo exon 1s (*UGT1BP, UGT1A11P, UGT1A12P,* and *UGT1A13P*) (Gong et al., 2001). UGT1 mRNAs are processed by differential splicing. The *UGT1* gene appears to be structurally distinctive, in that the UGT1 proteins result from differential splicing of the divergent first exons, and share exons 2-5 in common (Ritter, 1992).

A considerable number of UGT family polymorphisms exist. Human *UGT1A1* polymorphisms have been studied particularly closely (Maruo et al., 1999), because they are the causes of

hereditary unconjugated hyperbilirubinemias (Bosma et al., 1992; Aono et al., 1993; Sato et al., 1996) and are risk factors for neonatal hyperbilirubinemia and breast milk jaundice (Maruo et al., 1999, 2000). Polymorphisms of UGTs have also been found for *UGT1A3* (Iwai et al., 2004)*, UGT1A4* (Ehmer et al., 2004), *UGT1A6* (Ciotti et al., 1997)*, UGT1A7* (Guillemette et al., 2000)*, UGT1A8* (Huang et al., 2002)*, UGT1A10* (Jinno et al., 2003)*, UGT2B4* (Levesque et al., 1999), *UGT2B7* (Jin et al., 1993), and *UGT2B15* (Levesque et al., 1997).

Human UGT1A4, whose cDNA was first cloned by Ritter et al. (1991), has been identified in the human liver and stomach (King et al., 2000). It catalyzes glucuronidation of primary, secondary and tertiary amines, carcinogenic aromatic amines (ß-naphthylamine, 4-aminobiphenyl, and benzidine), androgens, progestins, and plant steroids (hecogenin, diosgenin, and tigogenin) (Green et al., 1995 Green and Tephly, 1996; King et al., 2000). UGT1A3 and UGT1A4 catalyze the glucuronidation of important endogenous substances including amines and steroids (Mojarrabi et al., 1996; Green et al., 1998). We have earlier described UGT1A3 polymorphisms in a Japanese population (Iwai et al., 2004). UGT1A4 is 93% identical to UGT1A3, and catalyzes the glucuronidation of a number of clinically important therapeutic agents including antihistamines, tricyclic

antidepressants, and antipsychotics such as clozapine (8-chloro-11-(4-methyl-1-piperazinyl)-5H-dibenzo[b,e][1,4]diazepine).

Clozapine is known to undergo a large number of biotransformation reactions in humans (Dain et al., 1997). Its metabolism is mediated by CYP1A2, CYP3A4 and CYP2D6 in the liver (Prior et al., 2003). When human liver microsomes are incubated with clozapine, at least 5% of the whole conjugates were the corresponding glucuronides (Breyer-Pfaff and Wachsmuth, 2001). In experiments with expressed human UGT1A4, clozapine was mainly conjugated to tertiary 5-N-glucuronides, and to a lesser extent to a quaternary ammonium (Luo et al., 1994; Green et al., 1995; Breyer-Pfaff and Wachsmuth, 2001). Glucuronidation of clozapine by other UGT isoformes has not been studied, and its biotransformation routes have not been identified in full. The therapeutic serum concentration of clozapine as an antipsychotic drug exceeds 350 ng/ml, and in animal experiments its hepatic concentration was 20 times higher than in the blood (Gauch and Michaelis, 1971). Clozapine also has an adverse effect in agranulocytosis, which is assumed to be mediated by a chemically reactive metabolite (Pirmohamed et al., 1995; Breyer-Pfaff and Wachsmuth, 2001).

Below, using clozapine as the substrate in gene expression

experiments, we analyzed the incidence and type of UGT1A4 polymorphisms in a Japanese population, and studied their activity.

Materials and Methods

Materials

[^{14}C]UDP-glucuronic acid (15.5GBq/mmol) was obtained from Dupont-NEN (Wilmington, DE, USA), and UDP-glucuronic acid and clozapine, trans-androsterone (5α-androstan-3ß-ol-17-one), imipramine (10,11-dihydro-N,N-dimethyl-5H-dibenz[b,f]azepine-5-propanamine hydrochloride), cyproheptadine (4-(5H-dibenzo[a,d]cyclohepten-5-ylidene)-1-methylpiperidine hydrochloride) and tigogenin ((25R)-5α-spirostan-3ß-ol) from Sigma-Aldrich (St. Louis, MO, USA). A human liver cDNA library, Mutant Km Kit, and pkF18 vector were obtained from TaKaRa (Kyoto, Japan), and pCR2.1 vector, pCR3.1 vector, and TA cloning kit from Invitrogen (San Diego, CA, USA). The thin layer chromatography (TLC) plastic sheet 5748 used below was obtained from Merck (Darmstadt, Germany), the Gene PORTER transfection reagent from Gene Therapy Systems (San Diego, CA, USA), the PVDF membrane from BIO-RAD (Hercules CA, USA), and the ECL Plus Western Blotting Detection Regents from Amersham Biosciences (Buckinghamshire, UK).

Sequence analysis of UGT1A4

Genomic DNA was isolated from the leukocytes of 100 healthy Japanese volunteers, with informed consent. The study was approved by the ethics committee of Shiga University of Medical Science. Exon 1 of *UGT1A4*, along with part of an intron, was amplified by PCR with primer pair 5'-TTTGTCTTCCAATTACATGC-3'/ 5'-AGATATGGAAGCACTTGTAAG-3'. The forward primer was located upstream of the initiation codon, and the reverse primer was in the intron. The 1075-bp product was amplified by PCR under the following conditions: initial denaturing for 5 min at 94˚C, followed by 1 min at 94˚C, 1 min at 62˚C, and 2 min at 72˚C for 30 cycles with a MiniCycler (MJ Research, USA). A final reaction for 8 min at 72˚C ensured complete extension of the PCR products. The amplified DNA fragment sequences were determined directly with a dRhodamine terminator FS Ready Reaction Kit and PRISM 310 (Perkin Elmer, USA). Sequencing primers were as follows:
5'-CCTTGAGTGTAGCCCAGCGT-3',
5'-AGGCGGTGGTCCTCACCCCA-3',
5'-TGCTGTGTTTTTTTGGAGGT-3',
5'-ATCTTGTCAGCTATGCATCC-3',

5’-CTCCAGGTTCCCCTGCCGCG-3’.

Analysis of allelic polymorphisms

We subcloned the PCR products (40 ng) to pCR 2.1 vectors (50 ng) using a TA-cloning kit. Transformation by the ligated products took place using a Competent High JM109 (Toyobo, Osaka, Japan).

Construction of expression vector

UGT1A4 DNA isolated from a human liver cDNA library was amplified by PCR with the primer pair. 5'-TTTGTCTTCCAATTACATGC-3'/ 5'-AGATATGGAAGCACTTGTAAG-3'. The cDNA was inserted into a pCR3.1 expression vector using a TA cloning kit. Mutation was induced by site-directed mutagenesis, using a Mutan Km Kit according to the manufacturer's instructions. The constructed cDNA was excised from the pCR3.1 vector by two restriction enzymes (*Pst* I and *BamH* I), and was ligated into a pkF18 vector (TaKaRa, Kyoto, Japan) for mutagenesis. To introduce the mutations, we used the following primer for the T to G transversion at nucleotide 142: 5’-

AGCTCCCGCACGGCCTCCCGC-3', where the mutation is indicated by underlining. After the substitution had been introduced into the pkF18 vector, the converted cDNA was cut out and re-ligated into the pCR3.1 vector. We checked the cDNA by sequencing.

Expression of UGT1A4 in COS-7 cells

Twenty-four hours before transfection, 6 x10^5 COS-7 cells suspended in Dulbecco's modified Eagle's medium with 10% fetal bovine serum were seeded onto 100-mm-diameter culture plates. For transfection, 5ml of Dulbecco's modified Eagle's medium containing 50 µl GenePORTER and 3.0 µg DNA was poured onto the cells. Four hours later, 5 ml of Dulbecco's modified Eagle's medium containing 20% fetal bovine serum was added to each plate. Two models were generated–wild type UGT1A4 and L48V UGT1A4–and nontransfected cells were used as the controls. After 48 hours, the transfected cells were harvested and stored at –80˚C prior to use. Protein content was measured with a BCA Protein Assay Kit (Pierce, Rockford, IL).

Preparation of antibody and measurement of expressed protein.

To preparation of a polyclonal antibody of UGT1A4, we chose a segment such that the amino acid sequence was unique among UGT1A4 isoforms and the mutation site (L48V) was excluded. We synthesized a 10-amino-acid segment (EHLLKRYSRS: residues 104-113) and generated its polyclonal antibody by immunizing rabbits. We purified the antibody by affinity chromatography of 18 ml of Thiol Sepharose 4B (Amersham Biosciences) coupled with 6 mg of the synthesized peptide. The column was equilibrated using Dulbecco's Phosphate Buffered Saline (Nacalai, Kyoto), and the antibody was eluted by 0.2 M-glycin-HCl buffer, pH 2.5. The final titer of the purified antibody was 1:51200.

Western blotting

Cell homogenates underwent sodium dodesyl sulfate-polyacrylamide gel electrophoresis. The protein was transferred to a PVDF membrane and visualized with ECL Plus Western blotting detection reagents. The membrane was incubated for 1 hour in blocking solution, 1 hour in a solution of anti-UGT1A4 antibody (1:500), and 1 hour in a solution of anti-rabbit antibody (1:15000).

The detection solution was added and the membrane was exposed to film for 5 min. The relative amounts of UGT1A4 expressed at the protein band peaks were measured with an Image Master-CL (Amersham Biosciences, Uppsala, Sweden). We blotted the wild type and L48V samples as a pair on the same membrane, and confirmed the measurements were in the linear range by assaying three different concentrations of an identical cell homogenate at the same time. The relative amount of L48V UGT1A4 was always within about 0.9-1.1 times that of wild type UGT1A4.

Assay of UGT1A4 activity

UGT1A4 activity was assayed as described previously, with a minor modification (Iwai et al., 2004). The glucuronidation process of the substrate was assayed with [^{14}C]UDP-glucuronic acid. The incubation mixture contained cell homogenate (100-150µg), clozapine (0-500µM), UDP-glucuronic acid (500µM), 9.25 kBq [^{14}C]UDP-glucuronic acid (5.97µM), DMSO (1%), $MgCl_2$ (10mM), 50mM Tris-HCl buffer (pH 8.4), and saccharolactone (8.5mM) in a final volume of 100 µl. Incubation took place at 37˚C for 30 min. The clozapine-glucuronide that resulted was isolated by thin layer chromatography (TLC) on TLC plastic sheet 5748. The TLC plates

were developed in a solvent system containing *n*-butanol, acetone, water, and ammonium hydroxide (35:35:20:10, v/v), and were scanned on an Instant Imager (Packard, Meriden, CT). Major and minor glucuronide metabolites of clozapine were detected, and their combined density was taken to represent the reaction product.

Under the same conditions, we measured the UGT1A4 activities of the wild type and L48V, using as substrates trans-androsterone (0-500µM), a tricyclic antidepressant, imipramine (0-500µM), an antihistamine, cyproheptadine (0-500µM), and tigogenin (0-100µM).

Data analysis

We calculated kinetic parameters using the Prism 3.0 software (Graph Pad Software, Inc., San Diego, CA, USA) and non-linear regression on the Michaelis-Menten equation. All data displayed are the means of three separate experiments. The enzyme efficiencies were subjected to a *t*-test for pairwise comparison.

Results

Identification of UGT1A4 mutations and their frequencies

We identified three nucleotide substitutions in exon 1 of *UGT1A4* in our Japanese test population. One was a T to G transversion at nucleotide position 142 (142T>G), that predicted a substitution of valine for leucine at codon 48 of the enzyme (L48V). The other two were novel, silent transitions (448T>C: L150L, 804G>A: P268P). We identified a novel substitution mutation in the intron (867+43C>T) and found no substitutions in exons 2-5. The incidence of the substitutions identified ranged from 0.155 to 0.165 (Table 1), indicating that they represented polymorphisms in the sample population. Indeed, analysis of subcloned exon 1 revealed the presence of four alleles, with frequencies ranging from 0.01 to 0.825 (Fig.1). Two of these (L48V-L150L-P268P-867+43C>T and P268P) were novel. We detected 5 patterns of allelic combinations (Fig.2).

Expression and identification of UGT1A4

In a Western blotting analysis, 55-kDa protein bands (Fig.3) (Miners and Mackenzie, 1991) were detected in all the expression models but not in the mock transfection; the molecular weight of the band was as reported previously.

Activities of UGT1A4 isoforms

Clozapine-glucuronide production was proportional to the amount of cell homogenate at up to 1000 µg. At 100 µg, the reaction product was linear with time up to 90 min. The reaction velocity increased steeply with pH and then reached a plateau at pH8.0-9.0. Lineweaver-Burk plots showed the apparent *Km* of the reaction to be 285µM at three clozapine concentrations (25µM, 50µM, and 200µM), and 66µM at three UDP-glucuronic acid concentrations (125µM, 250µM, and 500µM).

At about 200µM clozapine, glucuronidation by wild type UGT1A4 reached its maximum velocity and then declined, indicating substrate inhibition (Fig.4). Table 2 shows the kinetic parameters of the expressed wild type and L48V enzymes. The difference in *V*max between these was not statistically significant, and the *K*m of L48V was half that of the wild type enzyme, implying that the relative efficiency (*V*max/*K*m) of the homozygous model of L48V was twice

that of the wild type.

Table 3 shows kinetic parameters determined for UGT1A4 for trans-androsterone substrate, compareing that type for which the glucuronidation activity decreased in L48V with the wild type (Ehmer et al., 2004); comparisons are also presented for three other substrates, imipramine, cyproheptadine and tigogenin. The L48V had only 30-60% of *V*max value of the wild type for all four substrates. Apparent *K*m values of L48V for trans-androsterone, imipramine and cyproheptadine were also considerably smaller than for the wild type, but that of tigogenin was not changed. The L48V/wild ratios of efficiencies for trans-androsterone, imipramine, cyproheptadine and tigogenin were respectively 10.4, 1.8, 1.6 and 0.6.

Discussion

Glucuronidation is a major excretion pathway for diverse endobiotics and xenobiotics catalyzed by the UGT family of enzyme. A considerable number of UGT polymorphisms have been identified, some of which are associated with disease or adverse drug effects. Polymorphisms of *UGT1A1* are known to cause Gilbert syndrome, a mild hereditary unconjugated hyperbilirubinemia, breast milk jaundice (Maruo et al. 2000), and may contribute to breast cancer (Adegoke et al. 2004). Polymorphisms of *UGT1A4*, *UGT1A6*, *UGT1A7*, and *UGT1A8* may also be risk factors for carcinogenesis (Ehmer et al., 2004; Gagne et al., 2002; Strassburg et al., 2002). In our previous report we detected six novel *UGT1A3* SNPs and considered their possible contribution to differences between individuals in drug metabolism and susceptibility to side effects (Iwai et al., 2004). *UGT1A4* has a fairly similar amino acid sequence to *UGT1A3* and overlapping substrate specificity for several amines, suggesting the presence of its polymorphism.

In the experiments reported here, we analyzed *UGT1A4* in our Japanese test population. We identified two novel and one known single nucleotide substitution in exon 1, and one novel

substitution in intron 1 (Table 1). All incidences were greater than 0.01, indicating that the substitutions were polymorphisms. Recently, P24T and L48V polymorphisms have been detected in a Caucasian population in Germany (Ehmer et al., 2004). We did not detect P24T in our sample population, but L48V is a common polymorphism in Japanese as well as European populations. The present analysis showes that polymorphism of *UGT1A4* differs between races.

Linkage analysis of the alleles coded by the four *UGT1A4* SNPs revealed that their combinations generate three polymorphisms (Fig. 2), of which two (L48V-L150L-P268P-867 +43C>T and P268P) are novel. The amino acid sequence of the enzymes expressed by L48V-L150L-P268P-867 +43C>T and L48V were the same, but the former had an additional intronic polymorphism (867 +43C>T). Thus, based on amino acid sequences, there were only two enzymes in the sample population– wild type and L48V. The location of 867 +43C>T in the first intron was not in the typical consensus sequence of splicing, and the nature of its contribution, in the splicing of UGT1A4 mRNA is not clear.

In the present study, L48V was twice as efficient as the wild type in glucuronidating the antipsychotic drug clozapine (Table 2).

We also confirmed, by in vitro expression of the wild type enzyme, that the glucuronidation of clozapine shows substrate inhibition (Fig.4). Ehmer et al. (2004) reported that the L48V activity for trans-androsterone was lower than the wild type. Although our date also found a reduced *V*max of L48V for trans-androsterone (Table 3), the efficiency of L48V was 10 times higher than that of the wild type. Likewise, the efficiencies of L48V for imipramine and cyproheptadine were 1.6 – 1.8 times higher than that of the wild type, but that for tigogenin there was reduction by a factor of 0.6. These results indicate that L48V accelerates or slows the glucuronidation of UGT1A4, depending on substrates.

In conclusion, our results find a polymorphism of UGT1A4 (L48V) in Japanese population. An understanding of the effects of the mutation on the glucuronidation of substrate drugs would help clinicians who prescribe these drugs to calculate the appropriate dosage and avoid adverse effects.

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Footnotes

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Legends for figures

Fig. 1 *Amino acid substitutions found in UGT1A4 in the Japanese population sample, and their frequencies.*

Fig. 2 *Amino acid substitution combinations found in UGT1A4 in the Japanese population sample, and their frequencies.*

Fig. 3 *Identification of expressed UGT1A4 protein by Western blotting.*

A, Mock transfection; B, Wild type; C, L48V.

Fig. 4 *Velocity of reaction of wild type UGT1A4 with clozapine.* The reaction mixture contained 100 µg cell homogenate and 500 µM UDP-glucuronic acid, and was incubated for 30 min at pH 8.4. Note inhibition at >200µM clozapine.

Tables

Table 1 *Single nucleotide polymorphisms identified in exon 1 and intron 1 of UGT1A4*

Nucreotide position		Wild type	Mutant	Amino acid change	SNP frequency (n=200)
142	exon 1	T	G	L48V	0.165
448	exon 1	T	C	L150L (silent)	0.155
804	exon 1	G	A	P268P (silent)	0.165
867+43	Intron 1	C	T		0.155

Table 2 *Kinetic parameters of wild type and L48V UGT1A 4 for Clozapine*

Allele	*V*max[a] (pmol/min/mg)	*K*m[b] (μM)	*V*max/*K*m (μM/min/mg)
Wild type	1205±168.0	49.18±13.9	25.4±4.80
L48V	1315±246.8	25.41±5.05	52.7±13.1[c]

[a]*V*max: maximum velocity

[b]*K*m: Michaelis constant

[c]Significantly different from the wild type (*p<0.05). *P* value was obtained by using *t*-test

Table 3 *Kinetic parameters of wild type and L48V UGT1A4 for trans-androsterone, imiramine, cyproheptadine and tigogenin.*

Substrate		Wild type	L48V
Trans-androsterone	*V*max[a] (pmol/min/mg)	107.3	35.7
	*K*m[b] (μM)	254.7	8.2
Imipramine	*V*max (pmol/min/mg)	872.8	294.9
	*K*m (μM)	1442.0	275.8
Cyproheptadine	*V*max (pmol/min/mg)	342.6	203.3
	*K*m (μM)	227.5	86.4
Tigogenin	*V*max (pmol/min/mg)	158.6	81.4
	*K*m (μM)	7.8	6.7

[a]*V*max: maximum velocity

[b]*K*m: Michaelis constant

Fig.1

Amino Acid Alterations in UGT1A4	Mutant Frequency n=200
(Wild type)	0.825
L48V L150L P268P 867+43C > T	0.155
L48V	0.01
P268P	0.01

Fig.2

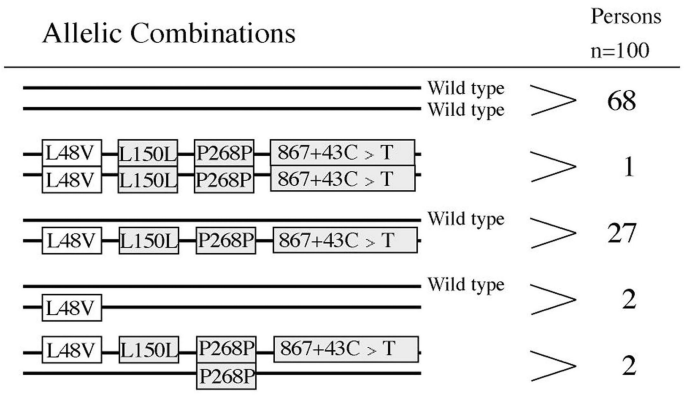

Allelic Combinations
Persons
n=100
Wild type
Wild type
68
L48V
L150L
P268P
867+43C > T
L48V
L150L
P268P
867+43C > T
1
Wild type
L48V
L150L
P268P
867+43C > T
27
Wild type
L48V
2
L48V
L150L
P268P
867+43C > T
P268P
2

Fig.3

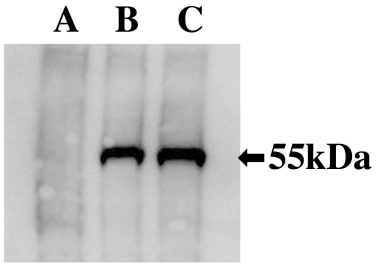
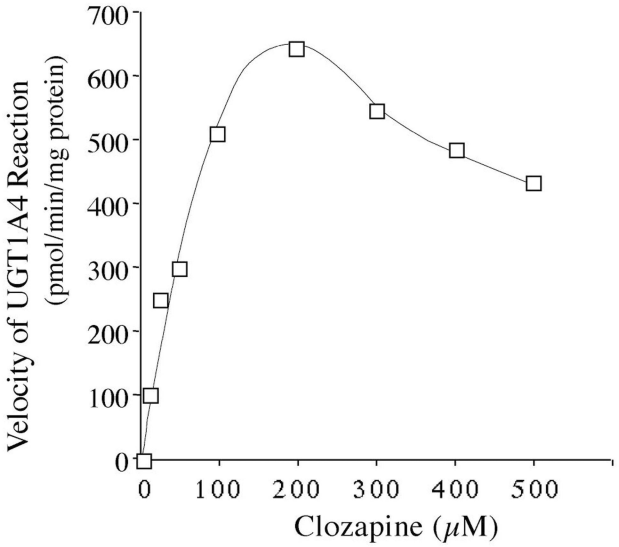


Fig.4


Velocity of UGT1A4 Reaction
(pmol/min/mg protein)
700
600
500
400
300
200
100
0
0
100
200
300
400
500
Clozapine (μM)