Comparison of Cytochrome P450 Mediated Metabolism of Three Central Nervous System Acting Drugs

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Abstract

Cytochrome P450s (CYPs) constitute one class of monooxygenases mainly responsible for oxidative transformation of xenobiotics in human, animals, and plants. Reactions catalyzed by CYPs are the initial steps leading to drug detoxification, aromatic and aliphatic hydroxylation, epoxidation, oxidative desulfuration, and sulfoxidation. However, these reactions can also result in intended or accidental drug activation. The CYP superfamily has been shown to play an important role in the oxidative metabolism of most (>90%) currently available drugs. Although many kinds of CYP proteins exist in human hepatic microsomes, CYP 2C, 2D6, and 3A4 are the isoforms that are responsible for metabolism for majority of drugs.

Drugs acting on the central nervous system (CNS) are of major therapeutic and clinical importance. They can produce diverse physiological and psychological effects such as relief of pain, reduction of anxiety, and treatment of depression. CNS drugs are classified into several classes which include anti-epileptics, anti-parkinsonism, analgesics, anesthetics, anti-psychotics, and anti-depressants. Although therapeutic range of these drugs is often narrow and hence clinical dose needs to be traced carefully, metabolism of CNS drugs is not fully understood.

In this paper, we report kinetic parameters for the metabolism of CNS-acting drugs, fluphenazine (FLU), dothiepin (DOT), and amitriptyline (AMI) (Fig. 1). FLU is one of the typical antipsychotic drugs belonging to the piperazine class of phenothiazines and is used in the treatment of schizophrenic disorder due to its neuroleptic effect. Some serious side effects have been reported for FLU such as akathisia, extra pyramidal side effects especially in case of long term therapy. FLU undergoes extensive first pass metabolism into large number of metabolites that include N4-oxide, sulfoxide, N-dealkylated derivatives, and ring-hydroxylated products such as 7-hydroxyfluphenazine.

On the other hand, DOT and AMI are tricyclic antidepressants, used for treatment of major depressive disorders. Some side effects have been reported for both of them such as drowsiness, dry mouth, and blurred vision. DOT is extensively metabolized after oral dosing into three major metabolites, northiaden, northiaden-oxide and dothiepin-5-oxide, while AMI is mainly metabolized by demethylation to desmethyiamitriptyline (nortriptyline) and didemethylamitriptyline, and also metabolized by aliphatic ring hydroxylation to 10-hydroxy amitriptyline and 10-hydroxy.

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Fig. 1. Chemical Structures of AMI, DOT, and FLU

AMI and DOT are classified as tricyclic antidepressants, while FLU belongs to piperazine class of phenothiazines.
The N-demethylation of AMI is described to be mainly governed by the CYP 2C19 gene as observed in both in vivo and in vitro experiments.\(^{20,25}\) Although the inhibitory effect of FLU on different CYP-substrates was studied before,\(^{17,26}\) and several metabolites have been identified on FLU and DOT as described above, the kinetic parameters for their metabolisms have never been reported yet.

Furthermore, we prepared three major CYP isoforms, 2C19, 2D6, and 3A4, and compared the parameters for the metabolism of three CNS drugs. It is because the CNS drugs could be metabolized differently by these isoforms and hence the metabolism may be affected differently by the polymorphisms of the CYP genes. It is apparent that variability in genes encoding drug metabolizing enzyme often affects individual drug response and adverse reaction to a great extent, and that the polymorphism of CYP genes plays a major role in this respect.

We first constructed purification procedures to obtain soluble CYP isoforms (2C19, 2D6 and 3A4) by using an Escherichia coli (E. coli) over-expression system which has several advantages including rapid growth, low cost, and high level of protein production. By using our system, we could find that 2C19 could be predominant for metabolic activity on AMI and DOT as expected, but FLU was almost equally metabolized by three CYPs. Our results strongly suggest that these CNS drugs are metabolized differently in vivo, and hence give a clue to clinical application of these commonly used CNS drugs.

**Experimental**

**Chemicals and Reagents** FLU and DOT, as well as recombinant human CYP reductase and cytochrome b\(_5\), were provided by Sigma Aldrich (St. Louis, U.S.A.). AMI and other chemicals were purchased from Wako (Osaka, Japan).

**Protein Expression and Purification** Genes encoding wild-type (WT) CYP 2C19, 2D6, and 3A4 were designed, synthesized, and ligated into pET3a-based expression vector (pBEX) as described previously.\(^{27,28}\) After 48h of culture in 2L of Terrific Broth medium, E. coli cells were harvested by centrifugation and resuspended in 100mL KPi buffer (100mM potassium phosphate, 0.1mM ethylenediaminetetraacetic acid (EDTA), 20% glycerol, pH 7.4), followed by sonication. The expressed CYPs were extracted by solubilization with lysozyme and CHAPS, and separated with ultracentrifugation (40000×g for 1h). The soluble fractions were passed through ion-exchange columns. The non-adsorbed fractions were loaded onto an octyl sepharose (GE Healthcare) column, and then red fractions were eluted with a gradient of Triton X-100 concentrations. If necessary, samples were further purified by hydroxyapatite column (BioRad). Finally, the buffer was completely replaced by 100mM KPi buffer by dialysis and then concentrated. The purified CYPs were confirmed and evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The CYP concentrations were determined by pyridine ferrohemochrome assay on the heme contents with a spectrophotometer (DU 800, Beckman Coulter).

**The Evaluation System on Kinetic Parameters** The kinetic parameters for drug metabolisms were measured using the purified CYP 2C19, 2D6, and 3A4 at various substrate concentrations from 25 to 400μM. The reactions were performed by mixtures of 0.1μM CYP (except for 3A4 which was 0.5μM), 0.4μM CYP reductase, 0.2μM cytochrome b\(_5\), 30μg/mL phosphatidyl choline, and substrates of certain concentrations. The concentration of phosphatidyl choline was referred to the previous study.\(^{29}\) We monitored metabolic activities using CYP reductase and cytochrome b\(_5\) under various conditions, and we identified these concentrations could be sufficient for the reaction. After pre-incubation of the 190μL mixture for 5min at 37°C, the reactions were initiated by additions of 10μL of a solution containing 1.0mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 2.5mM glucose 6-phosphate, 2.5mM MgCl\(_2\) and 2.0IU/mL glucose 6-phosphate dehydrogenase. The solutions were incubated at 37°C and samples were taken at interval of time. The reaction was stopped by the addition of equal volume of cold methanol. The precipitants were excluded by centrifugation at 14000rpm for 10min and then the supernatants (2–10μL) were directly injected into HPLC system as described below. Control experiments were performed in the absence of CYPs and we did not detect any changes in the content of target drugs as expected.

**HPLC Analysis** Measurements were carried out using HPLC LaChrom system (Hitachi, Japan) equipped with two pumps (L-2100), an auto sampler (L-2200), and a diode array detector (L-2455). The analytical column was TSK-GEL Super ODS column (4.6×100mm, TOSOH, Japan). The mobile phase consisted of 0.1% v/v acetic acid and acetonitrile in ratio of 67:33 for AMI, 61:39 for FLU, and 72:28 for DOT. Flow rates and column temperatures were set at 1.0mL/min and 40°C, respectively. UV detection wavelengths were at 240nm, 250nm, and 229nm for AMI, FLU, and DOT, respectively. The analytical conditions for HPLC were optimized individually to complete one run within 6.5min (AMI and FLU) and 8.5min (DOT). We carried out the calibration with different stock concentrations in order to check the solubility of each substrate in 100mM KPi buffer.

**Results**

We performed calibrations in order to identify the concentrations of stock solutions as mentioned, and the stock solutions that were completely soluble in 100mM KPi buffer were confirmed to be 1000, 400, and 600μM for AMI, FLU, and DOT, respectively. The data were analyzed by using Michaelis–Menten plots and Lineweaver–Burk plots, and the kinetic parameters, K\(_m\) and V\(_{max}\), as well as intrinsic clearance (CL\(_{int}\)), which is the ratio of V\(_{max}\) to K\(_m\) and is an index of metabolic efficiency, were obtained. Since each purified CYP was detected as a single major band by SDS-PAGE (Fig. 2), we could confirm that the CYPs were highly pure for evaluating kinetic parameters.

First, the kinetic parameters (K\(_m\), V\(_{max}\) and CL\(_{int}\)) of AMI were determined on CYP 2C19 (Fig. 3A, Table 1), and subsequently CYP 2D6 and 3A4 were also evaluated by the constructed method (Figs. 3B, C, Table 1). Graphical analysis of the data according to Lineweaver–Burk plots yielded linear plots (insets of Fig. 3). 2C19 was the most important enzyme mediating metabolism of AMI with a lowest K\(_m\) value (69μM) and a highest V\(_{max}\) value (15μM/min/μM of CYP). 2D6 exhibited a moderate metabolic activity on AMI (K\(_m\) 171μM and V\(_{max}\) 6.6μM/min/μM of CYP). In case of 3A4, however, we have performed experiments five times, and the data was slightly scattered because of the low activities for AMI. Hence, we took the averages of reaction rates and then plotted against the
concentration of AMI in order to estimate the kinetic parameters. CYP 3A4 displayed a minor metabolic activity with the smallest intrinsic clearance value \( CL_{int} = 20 \text{mL/min/µmol of CYP} \). Our data on the metabolism of AMI reached the same conclusion that was reported previously \(^{22}\), and hence we could confirm that our new system works nicely in evaluating drug metabolism by CYPs.

Subsequently, the kinetic parameters for DOT and FLU were determined on 2C19 (Fig. 4, Table 1), and we found that 2C19 exhibited a higher metabolic activity for DOT (which is a thio-analogue of AMI) with \( CL_{int} \) value evaluated as \( 126 \text{mL/min/µmol of CYP} \). On the other hand, 2C19 has a lower metabolic activity on FLU with \( CL_{int} \) value as \( 40 \text{mL/min/µmol of CYP} \). Then, we carried out our experiment in order to determine kinetic parameters for CYP 2D6 and 3A4 on both drugs (Fig. 5). 2D6 exhibited higher reaction velocity on DOT than on FLU without large difference in binding affinity of 2D6 on both of them. Therefore, 2D6 had a higher metabolic activity on DOT with \( CL_{int} \) value as \( 60 \text{mL/min/µmol of CYP} \).

Although 3A4 had a higher binding affinity for FLU, it exhibited a higher reaction velocity for DOT. Therefore, the calculated intrinsic clearance values for both of them were nearly the same (36, 31 mL/min/µmol of CYP for DOT and FLU, respectively). By comparison with other CYP isoforms (2C19 and 2D6), we could observe that 3A4 had the lowest metabolic activity on both of them.

### Discussion

The determination of kinetic parameters, \( K_m, V_{max} \), and \( CL_{int} \) for CYP-catalyzed reactions is an important aspect in drug discovery and development. In drug discovery, the data of the \textit{in vitro} study can be applied to drug designs in order to optimize the human pharmacokinetic behavior of new drugs, and it could also afford a useful tool in prediction of drug clearance prior to human administration. In drug development, the enzymatic parameters can contribute to interpreting the mechanism of clearance for new drugs in order...
to determine the inter-individual variability in pharmacokinetics such as supra proportional dose exposure relationship and also drug-drug interactions. For these reasons, we established an innovated and simplified method for measurement of metabolic activity on CYP isoforms for drugs by using purified CYPs which were provided by \textit{E. coli} over-expression system.

The system can produce enough amounts of CYPs suitable to investigate drug metabolisms \textit{in vitro}. These determinations were made using an approach of monitoring substrate depletion at various substrate concentrations from 25 to 400 $\mu$M.

Although the conventional methods reported previously\textsuperscript{33,34} were able to estimate the kinetic parameters for each CYP isoforms by measuring the formation of a certain metabolite, our innovated system has several advantages such as; we monitor the decrease in substrate concentration so it can provide the insight into effect of whole metabolic pathway in each CYP isoforms individually. This approach also affords reliable results without the need for information about the metabolites or requiring authentic samples of metabolites.\textsuperscript{35} Furthermore, we could obtain a highly purified CYP isoforms and hence we could avoid unexpected effect from any impurities that could be present in reaction mixture. In addition, since CYP 3A4 was easy to be inactivated and decomposed in the measurement for 40min, enzyme kinetic parameters were difficult to be estimated. In order to overcome this problem, we shortened the reaction time to 8min (40min for CYP 2C19 and 2D6) by using 5-fold higher concentration of CYP 3A4.

Subsequently, in order to evaluate our constructed method, we performed our experiment to estimate the kinetic parameters of CYP isoforms on several drugs which were previously reported, and we will show here AMI as a representative example. We measured the kinetic parameters for metabolism of AMI with three major isoforms, CYP 2C19, 2D6 and 3A4. We could estimate certain values which indicate that 2C19 has the smallest $K_m$ value and hence has a highest affinity to AMI, while the affinities of other CYPs were lower. Also, CYP 2C19 showed the highest $V_{\text{max}}$ and $CL_{\text{int}}$ for AMI in comparison with other CYP isoforms. Thus, we conclude that CYP 2C19 is the major isoform which is responsible for metabolism of AMI. This conclusion agree very well with other \textit{in vivo} studies which indicate that poor metabolizers with regard to (S)-mephenytoin (subjects lacking 2C19) have a remarkable decrease in metabolism of AMI.\textsuperscript{20} Also, another \textit{in vivo} study reported that 2C19 is an important determinant of plasma concentration of AMI and has highest capacity to demethylate AMI.\textsuperscript{21} In addition to these \textit{in vivo} studies, several \textit{in vitro} studies were carried out to determine the CYP isoform that is responsible for metabolism of AMI. All of these studies indicate that 2C19 is the major isoform mediating AMI metabolism.\textsuperscript{22,23} On the other hand, another \textit{in vivo} and \textit{in vitro} study reported that CYP 3A plays a relatively minor role in AMI clearance.\textsuperscript{36}

The reported kinetic parameters\textsuperscript{22,37–39} for demethylation of AMI are shown with interesting aspects (Table 1). Although our parameters for 3A4 are quite similar to those reported, the values for 2D6 are much disturbed. We suppose the difference can be attributed to the reaction condition and/or monitoring method. Subsequently, we calculated and simulated rate of metabolism in order to evaluate our data could reflect \textit{in vivo} experiments. The rate of metabolism for each isoform was calculated with the $K_m$ and $V_{\text{max}}$ values and an estimated drug concentration in hepatic tissues ($S$) according to the following Eq. 1,\textsuperscript{22} and the calculated values were summarized in Table 2.
Here, the populations of major CYP isoforms were reported, and the simulated rate of metabolism was finally evaluated as indicated. Consequently, the 2C19 could be the main metabolizer of AMI. Moreover, the estimated value also evoke that the 3A4 can play a relatively minor role in AMI clearance.

On the other hand, simulated rates in Table 2 predict that 3A4 plays a key role in the metabolism of DOT, although 2C19 also contributes significantly. In the case of FLU, 3A4 is a predominant metabolizer, although the rate is much lower than that for DOT. These results indicate that three CNS drugs are metabolized differently by three CYPs, and that DOT and FLU can be a possible substitute in case AMI induced an adverse effect in clinical use.

It is noteworthy that the metabolism of DOT and FLU has never been reported on their kinetic parameters, and the method could afford certain values for CYP 2C19, 2D6 and 3A4 as well. We found that 2C19 has a highest metabolic activity on DOT and hence we can conclude that 2C19 could be the major isoform mediating metabolism of tricyclic antidepressant drugs in vitro (as AMI and DOT). Therefore, the occurrence of single nucleotide polymorphisms (SNPs) on CYP 2C19 will have a major effect on tricyclic antidepressants in vivo, leading to low/high drug level in human plasma and a greater effect on drug efficacy and toxicity.

On the other hand, 2D6 has a higher \( V_{\text{max}} \) value on DOT than that on AMI with nearly the same \( K_m \) values for both. It could be explained as 2D6 could be responsible for DOT sulfoxidation (the metabolic pathway which is not involved in AMI metabolism). Therefore, 2D6 has a higher metabolic activity on DOT in comparison with AMI, although its contribution to the DOT metabolism may be minor in vivo (Table 2).

Although \( K_m \) and \( V_{\text{max}} \) values of 3A4 on DOT are different from that on AMI, 3A4 has a lowest metabolic activity on both drugs. The difference in \( K_m \) and \( V_{\text{max}} \) values could be due to the presence of sulfur atom in DOT (instead of benzylic CH\(_2\) in AMI), as substitution of benzylic carbon in AMI with sulfur atom in DOT changes the geometry of the molecules accompanied with decreasing the rate of bridge flexing motion of the central ring, making distortion of the planarity of DOT structure. This distortion may have a great effect on binding affinity and maximum reaction capacity of DOT compared with AMI.

Table 2. Simulated Rate of Drug Metabolism in Human Liver

<table>
<thead>
<tr>
<th>CYP isoform</th>
<th>Populations of CYPs in human liver (%)(^a) (^b)</th>
<th>Drugs</th>
<th>Rate of metabolism in vitro ((\mu\text{mol} / \mu\text{mol CYP}))</th>
<th>Simulated rate of metabolism in vivo ((\mu\text{mol} / \mu\text{mol CYP}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>2C19</td>
<td>5.0</td>
<td>AMI</td>
<td>114</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DOT</td>
<td>38</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FLU</td>
<td>3.0</td>
<td>0.15</td>
</tr>
<tr>
<td>2D6</td>
<td>1.5</td>
<td>AMI</td>
<td>22</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DOT</td>
<td>19</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FLU</td>
<td>3.7</td>
<td>0.051</td>
</tr>
<tr>
<td>3A4</td>
<td>30</td>
<td>AMI</td>
<td>11</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DOT</td>
<td>11</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FLU</td>
<td>2.3</td>
<td>0.70</td>
</tr>
</tbody>
</table>

\( a \) The values were calculated by Eq. 1. The drug concentrations in hepatic tissues \( (S) \) were estimated to be 20 times higher\(^c\) than those in plasma (45 ng/mL for DOT\(^d\), 27.9 ng/mL for FLU\(^e\)). Accordingly, \( S \) values were estimated to be 10 \( \mu \)mol (AMI)\(^f\), 5.4 \( \mu \)mol (DOT), and 1.2 \( \mu \)mol (FLU). \( b \) The values were estimated according to the populations of CYPs in human liver.\(^g\)

FLU showed different aspects on the metabolic activity. It could be related to the difference in the chemical structure of FLU. Hence, the three isoforms have no significant difference in the values of intrinsic clearance, and the values suggested that CYP 2C19, 2D6 and 3A4 can contribute infrequently to metabolism of FLU.

In this work, we have investigated the metabolism of three drugs acting on CNS in vitro. Although AMI and DOT indicated similar aspects for metabolisms, FLU showed significant difference to the isoforms. FLU is related to piperazine class with a phenothiazine ring, while AMI and DOT are in tricyclic antidepressant class. We identified that CYP 2C19 has a higher intrinsic clearance on drugs belonging to the tricyclic antidepressant class than that in piperazine class and that CYP 2D6 has a moderate one on both classes.

In vivo, our study could be helpful during clinical treatment with such CNS drugs. As generally, polymorphism in the CYP2C19 genes affects the metabolism of several classes of drugs. Individuals can be classified as extensive metabolizers or poor metabolizers on the basis of their ability to metabolize 2C19 substrates. Poor metabolizers carrying defective CYP2C19 gene are present at a frequency of up to 5% in Caucasian and African population and approximately 20% in Asians. Based on our results, we could conclude that tricyclic antidepressants (such as AMI and DOT) should be contraindicated for treatment of depression in poor metabolizers due to the reduced metabolism of these drugs in such individuals, thus leading to increase in drug plasma levels resulting in a serious side effect and even toxicity during drug therapy. While FLU (as a representative example of piperazinyl phenothiazines antipsychotics) could be a drug of choice for treatment of schizophrenic disorder in such individual, due to minor metabolic activity of 2C19 on this class of drugs. In addition, DOT and FLU is less affected by SNP mutation of 2C19, and hence these drugs can be a substitute for AMI. This could be helpful during clinical treatment especially to Japanese patient, because SNP mutations in 2C19 occur frequently in Japanese population.

In conclusion, we could establish an innovated and simplified in vitro method for measurement of metabolic activity of CYP isoforms and could report kinetic parameters of two new CNS acting drugs, DOT and FLU. Also, in these studies, we could provide the basis to understand metabolism of some CNS drugs at the molecular level as these drugs constitute one of the most clinically important drug classes, and we would
be able to propose a way of clinical usage of these drugs to patients who are poor or extensive metabolizers in respect to 2C19. In addition, our simple and innovative method can be applied for determination of major CYP isoforms which are responsible for metabolism of new drugs, and it also can be introduced for studying the effect of single-nucleotide polymorphisms on drug metabolism.

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