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Application of Microtiter Plate Assay to Evaluate Inhibitory Effects of Various Compounds on Nine Cytochrome P450 Isoforms and to Estimate their Inhibition Patterns

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Summary: Using a microtiter plate (MTP) assay consists of recombinant cytochromes P450 and fluorescent probes, we evaluated inhibitory effects of commercially available model-compounds, 18 typical substrates and 8 selective inhibitors, on nine cytochromes P450 (CYPs) activities. The IC50 values obtained from the assay were used to estimate inhibition constant (Ki) values, assuming competitive inhibition. The Ki values calculated from IC50 (the Ki-cal) with the MTP assay using recombinant CYPs were compared with the Ki values (the Ki-rep), reported for human liver microsomes (HLM). Regarding all the inhibitory effects of the 26 test compounds on each CYP activity, a good correlation (r^2 = 0.7306) was found between Ki-cal and Ki-rep.

The inhibitory patterns of some compounds on the five major CYP isoforms were estimated, using the MTP assay with the preincubation method. Furafylline and erythromycin, both mechanism based inhibitors, strongly inhibited CYP1A2 and CYP3A4 activity, respectively and their inhibitory effects increased depending on the preincubation time. In contrast, the inhibitory effects of phenacetin, diclofenac, S-mephenytoin, dextromethorphan, bufuralol and terfenadine, typical substrates for CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4, respectively, on each recombinant CYP activity decreased after preincubation.

Therefore, the MTP assay is a useful high throughput screening method to evaluate inhibitory effects of new drug candidates on 9 CYP isoforms in HLM. In addition, the MTP assay with the preincubation method might be beneficial to estimate inhibitory patterns on CYP isoforms of new drug candidates and to estimate main CYP isoforms responsible for metabolism of these compounds.

Key words: cytochrome P450; enzyme inhibition; high throughput screening; microtiter plate assay

Introduction

Recently, in many pharmaceutical industries, high throughput screening (HTS) methods to determine drug metabolism and pharmacokinetics (PK) have been required to adapt to the rapid development of combinatorial chemistry and HTS for pharmacological efficacy at the stage of early drug discovery. The recent focus on the drug metabolism and PK studies is to select and to optimize the compound possessing good PK profiles at the stage of early drug discovery, because many failures due to pharmacokinetic problems became apparent at the time in clinical stage and marketing for the several ten years. Avoiding a compound that has metabolic instability and that inhibits metabolic enzyme is a global standard in the drug metabolism studies.

Since cytochromes P450 (CYPs) catalyzing the oxidative metabolism of many xenobiotics and drugs play very important roles in drug metabolism, human liver microsomes (HLM) and recombinant CYPs are usually used to assess metabolic stability and inhibitory effects. The metabolic stability of new drug candidates by HLM has been generally measured using liquid chromatography/mass spectrometry (LC/MS/MS) analysis at the stage of drug discovery. In vitro intrinsic clearance (CLint), which is calculated from the disappearance
rate of an unchanged drug, has generally served as a parameter of metabolic stability. However, even if the 96 well plate incubation-formats and LC/MS/MS analysis are used to evaluate metabolic stability and inhibitory effects, such evaluations are time- and labor-consuming for measurements of unchanged drug or CYP marker metabolites.

Recently, new methods for inhibition studies were developed as HTS to evaluate the inhibitory effect of new drug candidates. Some of those are methods using the combination of solid phase extraction and LC/MS/MS or using that of cocktail of substrates and LC/MS/MS simultaneous analysis. Others methods involve use of a recombinant CYP and fluorescent probe or radiometric probe. Especially, the microtiter plate (MTP) assay using recombinant CYP and a fluorescent probe has predominantly been used in many pharmaceutical laboratories to evaluate inhibitory effects of new drug candidates. The principle of this method is to measure the fluorescence of a metabolite from a typical substrate. Therefore, the throughput of the MTP assay remarkably increased in comparison with conventional methods, using HPLC analysis or LC/MS/MS simultaneous analysis. In general, HLM is used for inhibition studies, but recombinant CYP microsomes are rarely used in the MTP assay, because most of the substrates used in the MTP assay are not CYP-specific. Therefore, the important point in this assay is to confirm if inhibitory effects of compounds on recombinant CYP reflect those on HLM.

In a recent study, the reliability of this method was confirmed by comparing inhibitory effects (IC_{50} values) of some compounds on recombinant CYPs and those on HLM. However, since IC_{50} values depend on concentration of the substrate used, a comparison of the Ki value among new drug candidates is more important than that of the IC_{50} value. Bapiro et al. reported that Ki values calculated from IC_{50} obtained by MTP assay agree well with Ki values observed from traditional HPLC-based assays in HLM with regard to CYP1A2 and CYP2D6, but more many data are needed to support this evaluation.

The primary purposes of our study were to confirm that the Ki values, a most important parameter regarding prediction of drug-drug interactions, can be readily estimated from the MTP assay and that the Ki from the recombinant reflects Ki values from HLM. Our secondary aim was to confirm that the MTP assay with the preincubation method is applicable to estimation of inhibitory patterns (particularly; competitive inhibition as substrate metabolism or mechanism based inhibition) by test compounds regarding five recombinant CYPs and that this assay is a useful HTS method to identify metabolic properties of new drug candidates at the stage of early drug discovery.

### Materials and Methods

**Chemicals:** 3-Cyano-7-ethoxycoumarin (CEC), 3-cyano-7-hydroxycoumarin (CHC) and 7-ethoxy-4-trifluoroethyl-coumarin (EFC) were purchased from Molecular Probe (Eugene, OR, USA). 7-Hydroxy-4-trifluoroethylocoumarin (HFC), dibenzylfluorescein (DBF), 7-methyl-4-trifluoromethyl-coumarin (MFC), 3-[2-N,N-diethyl-N-methylamino]ethyl]-7-methyl-4-ethoxy-4-methyl-coumarin (AMMC), 3-[2-(N,N-diethylamino)-ethyl]-7-hydroxy-4-methyl-coumarin (AHMC), 7-benzyloxyquinoline (7-BQ) and 7-hydroxyquinoline were purchased from Gentest Co. (Woburn, MA, USA). Furafylline, sulfaphenazole, S-mephenytoin, quinidine, bufuralol and ketoconazole were purchased from Ultrafine Chemicals Co. (Manchester, UK). Coumarin, 7-hydroxy coumarin, troleandomycin (TAO), tranylcypromine, quercetin, diethylidithiocarbamic acid (DDTC), imipramine, taxol, α-naphthoflavone, tolbutamide, lansoprazole, erythromycin, terfenadine, dextromethorphan, theophylline and fluorescein were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Phenacetin, pilocarpine, diclofenac, carbamazepine, p-nitrophenol, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and NADP^{+} were purchased from Wako Pure Chemical Industries (Osaka, Japan). Clarithromycin (CAM) and compound A were synthesized in Taisho Pharmaceutical Co. Ltd. (Saitama, Japan). All other chemicals and solvents used were of the highest quality commercially available.

**Enzymes:** Microsomes from bacuvirus-insect cells expressing human CYP isoforms, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 (recombinant CYPs microsomes), were purchased from Gentest Co. (Woburn, MA, USA).

**Enzyme inhibition assay:** The effect of eighteen typical substrates and eight specific inhibitors for each CYP isoform on nine human cytochrome P450 activities (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4) were evaluated using the microtiter plate (MTP) assay described by Crespi et al. Namely, this assay was done using a 96 well (final volume 200 μl) microtiter plate (Corning Coster, Cambridge, MA, USA) based on the method described on the Gentest Corporation website (http://www.gentest.com). Details are summarized in Table 1. Substrates used in this inhibition study were as follows: CEC (CYP1A2, CYP2C19), coumarin (CYP2A6), EFC (CYP2B6), DBF (CYP2C8), MFC (CYP2C9, CYP2E1), AMMC (CYP2D6) and 7-BQ (CYP3A4). Furafylline, α-naphthoflavone, theophylline and phenacem were used for inhibition studies on CYP1A2, tranylcypromine and pilocarpine were used for CYP2A6, tranylcypromine and DDTC were used for CYP2B6, querc-
Table 1. Experimental condition of microtiter plates assay for inhibition study on each cytochrome P450 activity

<table>
<thead>
<tr>
<th>Cytochrome P-450 Enzyme</th>
<th>CYP1A2</th>
<th>CYP2A6</th>
<th>CYP2B6</th>
<th>CYP2C8</th>
<th>CYP2C9</th>
<th>CYP2C19</th>
<th>CYP2D6</th>
<th>CYP2E1</th>
<th>CYP3A4</th>
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<tbody>
<tr>
<td>K-Phosphate-Buffer (pH 7.4)</td>
<td>100 mM</td>
<td>—</td>
<td>100 mM</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>100 mM</td>
<td>100 mM</td>
<td>200 mM</td>
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<tr>
<td>Tris pH 7.5</td>
<td>—</td>
<td>100 mM</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Substrate (concentration)</td>
<td>CEC</td>
<td>Coumarin</td>
<td>EFC</td>
<td>DBF</td>
<td>MFC</td>
<td>CEC</td>
<td>AMMC</td>
<td>MFC</td>
<td>7-BQ</td>
</tr>
<tr>
<td>Metabolite</td>
<td>CHC</td>
<td>3 μM</td>
<td>2.5 μM</td>
<td>1 μM</td>
<td>75 μM</td>
<td>25 μM</td>
<td>1.5 μM</td>
<td>100 μM</td>
<td>40 μM</td>
</tr>
<tr>
<td>Enzyme (content/well)</td>
<td>0.5 pmol</td>
<td>1.0 pmol</td>
<td>1.0 pmol</td>
<td>4.0 pmol</td>
<td>1.0 pmol</td>
<td>0.5 pmol</td>
<td>1.5 pmol</td>
<td>2.0 pmol</td>
<td>3.0 pmol</td>
</tr>
<tr>
<td>Fluorescent Excitation (bandwidth: nm)</td>
<td>409(20)</td>
<td>390(20)</td>
<td>409(20)</td>
<td>485(20)</td>
<td>409(20)</td>
<td>409(20)</td>
<td>390(20)</td>
<td>409(20)</td>
<td>409(20)</td>
</tr>
<tr>
<td>Emission (bandwidth: nm)</td>
<td>460(40)</td>
<td>460(40)</td>
<td>530(25)</td>
<td>538(25)</td>
<td>530(25)</td>
<td>460(40)</td>
<td>460(40)</td>
<td>530(25)</td>
<td>530(25)</td>
</tr>
<tr>
<td>Incubation time</td>
<td>15 min</td>
<td>15 min</td>
<td>30 min</td>
<td>30 min</td>
<td>45 min</td>
<td>30 min</td>
<td>45 min</td>
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<tr>
<td>Stop solution</td>
<td>MeCN</td>
<td>MeCN</td>
<td>MeCN</td>
<td>NaOH</td>
<td>MeCN</td>
<td>MeCN</td>
<td>MeCN</td>
<td>MeCN</td>
<td>MeCN</td>
</tr>
</tbody>
</table>

Concentrations shown are final concentration of each reagent in the incubation medium.

tin, taxol and carbamazepine were used for CYP2C8, sulphafenazole, tolbutamide, phenytoin and diclofenac were used for CYP2C9, tranylcypromine, S-mephenytoin, lansoprazole and imipramine were used for CYP2C19, quinidine, dextromethorphan, imipramine and bufuralol were used for CYP2D6, DDTC and p-nitrophenol were used for CYP2E1, ketoconazole, tranylcypromine, S-mephenytoin, lansoprazole, terfenadine erythromycin and clarithromycin (CAM) were used for CYP3A4.

All test compounds except for coumarin, tranylcypromine (used for CYP2B6 inhibition) and CAM were dissolved in acetonitrile. Coumarin, tranylcypromine and CAM were dissolved in de-ionized water, 25% acetonitrile and 50% acetonitrile, respectively. Final concentration ranges of inhibitors, positive control, were as described on the Gentest website. All test compounds except for pilocarpine, CAM and S-mephenytoin were examined in the range from 0.09 to 200 μM as the final concentration. Pilocarpine and CAM were tested in the range from 0.05 to 100 μM, and S-mephenytoin was tested in the range from 0.23 to 500 μM. The final acetonitrile concentration was 2%. The final concentrations of substrate (AMMC) for CYP2D6 were 0.75, 1.5, 3 and 6 μM. Serial dilution of quinidine and additions of all reagents to the 96 well plates were done by manual pipetting.

After incubation mixtures containing the serially diluted quinidine and NADPH-generating solution were pre-warmed at 37°C for 10 min, the reaction was initiated by adding 100 μL of CYP2D6/AMMC mixtures. After 30 min, the reaction was stopped by adding 80% acetonitrile/tris buffer. The determination of fluorescence in each well was done as described above. The inhibition constant (the Ki<sub>obs</sub>) was observed by a secondary plot of K<sub>m</sub>/V<sub>max</sub> versus inhibitor concentration from Lineweaver-Burk plots for experiments consisting of 6 concentrations of the inhibitor and 4 concentrations of the substrate. The IC<sub>50</sub> values of quinidine were obtained from same experiments with one concentration (1.5 μM) of AMMC. The Ki<sub>calc</sub> value of quinidine was calculated using the equation described for Data analysis. The Ki<sub>calc</sub> value was compared with the Ki<sub>obs</sub> value.

Estimation of time-dependent inhibition using the preincubation method: With respect to CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4, we studied effects of preincubation on the inhibitory potential of the test compounds (furafylline, α-naphthoflavone, phenacetin, sulphafenazole, diclofenac, phenytoin, tranylcypromine, S-mephenytoin, lansoprazole, quinidine, dextromethorphan, bufuralol, ketoconazole, erythromycin, terfenadine and compound A) for each fluorescent metabolic activity using the MTP assay with minor modifications. The concentration of test com-

Effect of quinidine on recombinant CYP2D6 microsomes: For quinidine, which is a competitive inhibitor of CYP2D6 activity, we determined the inhibitory pattern, using Lineweaver-Burk plots. This study was done using 96 well plates. The concentrations of quinidine were in the range from 0.0008 to 0.01 μM, as the final concentration. The final acetonitrile concentration was 2%. The final concentrations of substrate (AMMC) for CYP2D6 were 0.75, 1.5, 3 and 6 μM. Serial dilution of quinidine and additions of all reagents to the 96 well plates were done by manual pipetting.

After incubation mixtures containing the serially diluted quinidine and NADPH-generating solution were pre-warmed at 37°C for 10 min, the reaction was initiated by adding 100 μL of CYP2D6/AMMC mixtures. After 30 min, the reaction was stopped by adding 80% acetonitrile/tris buffer. The determination of fluorescence in each well was done as described above. The inhibition constant (the Ki<sub>obs</sub>) was observed by a secondary plot of K<sub>m</sub>/V<sub>max</sub> versus inhibitor concentration from Lineweaver-Burk plots for experiments consisting of 6 concentrations of the inhibitor and 4 concentrations of the substrate. The IC<sub>50</sub> values of quinidine were obtained from same experiments with one concentration (1.5 μM) of AMMC. The Ki<sub>calc</sub> value of quinidine was calculated using the equation described for Data analysis. The Ki<sub>calc</sub> value was compared with the Ki<sub>obs</sub> value.

Estimation of time-dependent inhibition using the preincubation method: With respect to CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4, we studied effects of preincubation on the inhibitory potential of the test compounds (furafylline, α-naphthoflavone, phenacetin, sulphafenazole, diclofenac, phenytoin, tranylcypromine, S-mephenytoin, lansoprazole, quinidine, dextromethorphan, bufuralol, ketoconazole, erythromycin, terfenadine and compound A) for each fluorescent metabolic activity using the MTP assay with minor modifications. The concentration of test com-

...
Fig. 1. Inhibitory effects of 26 test compounds on nine recombinant human CYPs activities. Inhibition study using MTP assay was carried out as described in Materials and Methods. Concentrations shown in parentheses represent final concentration in the incubation medium of each fluorescent probe used as substrate. Test compounds used as inhibitor in this study are 18 typical substrates and 8 selective inhibitors. Each point is mean of duplicate or triplicate determinations.

Data analysis: The constitution activity of the individual fluorescence metabolism product was proportional to the incubation time. Data were exported and analyzed using an Excel spreadsheet (Microsoft, USA). The inhibitory effect at each concentration of inhibitor or test compound on the specific activity was expressed as a percentage of the control value. The IC50 values were calculated by linear interpolation. The inhibition constant (the $K_i$-cal) values were calculated from IC50 values, assuming competitive inhibition, according to the following relationship, which is described by Bapiro et al., $K_i = (K_m \cdot IC_{50})/(K_m + S)$, where $K_m$ and S are affinity constants, respectively for each metabolic activity and substrate concentration used in this study.

In this study, we used this equation for all the compounds tested. This is because competitive inhibition is the most general inhibition pattern observed in the drug-drug interactions. In addition, the objective of the present study was to more readily estimate the degree of inhibition ($K_i$ values) using the MTP assay as HTS. Therefore, we used the above equation, assuming competitive inhibition for all compounds.

Results

Correlation between the $K_i$-cal and $K_i$-rep values: We evaluated effects of 26 test compounds on each fluorescence metabolic activity for nine CYP isoforms, using the MTP assay. The test compounds used in this inhibition study were 8 inhibitors as a positive control and 18 typical substrates for each CYP isoform known to exist in humans. Figure 1 shows the relationship between residual activity and test compound concentration with...
respect to nine CYP isofoms. Inhibitory patterns of all test compounds except for theophylline, taxol and carba
mazepine (used for the inhibition of CYP1A2 and CYP2C8) exhibited sigmoid curves in the range of concen
trations used.

As shown in Table 2, the IC\textsubscript{50} values of a typical sub
strate for each CYP isoform obtained, using the MTP assay were 70.14 ± 13.46 \mu M (CYP1A2: phenacetin), 15.07 ± 4.49 \mu M (CYP2A6: pilocarpine), 126.95 \mu M (CYP2B6: DDTc), >200 \mu M (CYP2C8: carbamazepine), 2.80 \mu M (CYP2C9: diclofenac), 165.12 \mu M (CYP2C9: phenacetin), 192.36 \mu M (CYP2C19: S-mephenytion), 37.54 \mu M (CYP2C19: imipramine), 37.54 \mu M (CYP2D6: imipramine), 4.46 \mu M (CYP2D6: bufuralol), 32.4 ± 5.48 \mu M (CYP3A4: tolbutamide), 162.21 ± 79.22 \mu M (CYP3A4: terfenadine), 11.82 \mu M (CYP3A4: erythromycin), 0.13 \mu M (CYP3A4: theophylline), 1.26 \mu M (CYP3A4: tolbutamide, maximum concentration as inhibitor used in this study was 200 \mu M). Since CYP2C9 activity was inhibited by 45% at the concentra
tion of tolbutamide as shown in Fig. 1, we used 200 \mu M instead of the IC\textsubscript{50} value to calculate the Ki\textsubscript{cal}. For clarithromycin (CAM), maximum concentration as inhibitor used in this study was 100 \mu M. CAM at the maximum concentration inhibited CYP3A4 activity by 34% and the inhibitory effect of CAM was smaller than that of erythromycin at same inhibitor concentration (Fig. 1). Therefore, we used 200 \mu M instead of the IC\textsubscript{50} value to calculate the Ki\textsubscript{cal} of CAM.

a) Ki values were estimated from the IC\textsubscript{50} for each recombinant CYP obtained with the MTP assay using the following equation; \( K_i = (K_m \times IC_{50}) / (K_m + S) \)

b) These values (the Ki\textsubscript{cal}) are the inhibition constant observed from human liver microsomes reported in the literatures.

c) Although the IC\textsubscript{50} values could not be obtained in this study, the Ki\textsubscript{cal} was calculated by application of 200 \mu M instead of the IC\textsubscript{50}. For tolbutamide, maximum concentration as inhibitor used in this study was 200 \mu M. Since CYP2C9 activity was inhibited by 45% at the concentration of tolbutamide as shown in Fig. 1, we used 200 \mu M instead of IC\textsubscript{50} to calculate the Ki\textsubscript{cal}. For clarithromycin (CAM), maximum concentration as inhibitor used in this study was 100 \mu M. CAM at the maximum concentration inhibited CYP3A4 activity by 34% and the inhibitory effect of CAM was smaller than that of erythromycin at same inhibitor concentration (Fig. 1). Therefore, we used 200 \mu M instead of the IC\textsubscript{50} value to calculate the Ki\textsubscript{cal} of CAM.

d) This is the K\textsubscript{m} value of the compound for each CYP isoform.

e) Since theophylline, carbamazepine and taxol hardly inhibited CYP1A2 and CYP2C8 activities at maximum concentration used, we did not calculate the Ki\textsubscript{cal}.

f) This is IC\textsubscript{50} value. All experiments were done in duplicate or triplicate. The results from three experiments represent as mean ± S.D.

### Table 2. Comparison of Ki\textsubscript{cal} values from MTP assay and the Ki\textsubscript{rep} values in the literatures

<table>
<thead>
<tr>
<th>CYP isoforms</th>
<th>Substrates or Inhibitors</th>
<th>IC\textsubscript{50} (\mu M)</th>
<th>Ki (\mu M)</th>
<th>Calculated\textsuperscript{a)}</th>
<th>Reported\textsuperscript{b)}</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A2</td>
<td>Phenacetin</td>
<td>70.14 ± 13.46</td>
<td>28.88</td>
<td>30.00</td>
<td>27) Campbell et al., (1987)</td>
<td></td>
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<tr>
<td></td>
<td>Furafylline</td>
<td>1.56 ± 0.21</td>
<td>0.64</td>
<td>0.70</td>
<td>22) Bourrie et al., (1996)</td>
<td></td>
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<tr>
<td></td>
<td>α-Naphthoflavone</td>
<td>0.02</td>
<td>0.01</td>
<td>0.01</td>
<td>22) Bourrie et al., (1996)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Theophylline</td>
<td>&gt;200</td>
<td>—</td>
<td>800.00</td>
<td>27) Campbell et al., (1987)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pilocarpine</td>
<td>15.07 ± 4.49</td>
<td>4.31</td>
<td>4.00</td>
<td>22) Bourrie et al., (1996)</td>
<td></td>
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<td>2A6</td>
<td>Tranylcypromine</td>
<td>0.43 ± 0.05</td>
<td>0.12</td>
<td>0.17</td>
<td>14) Taavitsainen et al., (2001)</td>
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<td></td>
<td>Dextromethorphan</td>
<td>1.89 ± 0.13</td>
<td>0.16</td>
<td>0.11</td>
<td>31) Leemann et al., (1993)</td>
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<td></td>
<td>Sulfaphenazole</td>
<td>0.31 ± 0.01</td>
<td>0.01</td>
<td>0.11</td>
<td>32) Doecke et al., (1991)</td>
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<td></td>
<td>Phenytoin</td>
<td>165.12</td>
<td>84.18</td>
<td>50.00</td>
<td>33) Veronese et al., (1991)</td>
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<td>2C9</td>
<td>Diclofenac</td>
<td>2.80</td>
<td>1.43</td>
<td>1.40</td>
<td>30) Tang et al., (2000)</td>
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<td></td>
<td>S-mephenytoin</td>
<td>192.36</td>
<td>103.30</td>
<td>50.80</td>
<td>34) Bort, R. et al., (1999)</td>
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<td></td>
<td>Imipramine</td>
<td>37.54</td>
<td>20.16</td>
<td>12.50</td>
<td>35) Chiba et al., (1994)</td>
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<td></td>
<td>Lansoprazole</td>
<td>0.36</td>
<td>0.30</td>
<td>3.20</td>
<td>36) Ko et al., (1997)</td>
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<tr>
<td></td>
<td>Tranylcypromine</td>
<td>1.31 ± 0.13</td>
<td>0.70</td>
<td>8.70</td>
<td>37) Wienkers et al., (1996)</td>
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<td></td>
<td>Dextromethorphan</td>
<td>1.89 ± 0.13</td>
<td>0.757</td>
<td>2.70</td>
<td>38) Broly, F. et al., (1990)</td>
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<td>Impiramine</td>
<td>2.71 ± 0.46</td>
<td>1.086</td>
<td>3.90</td>
<td>39) Ball et al., (1997)</td>
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<td>Quinidine</td>
<td>0.01 ± 0.001</td>
<td>0.003</td>
<td>0.005</td>
<td>40) Wu et al., (1993)</td>
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<td>2D6</td>
<td>P-Nitrophenol</td>
<td>34.23 ± 5.48</td>
<td>22.82</td>
<td>42.00</td>
<td>42) Tassaneeyakul et al., (1993)</td>
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<tr>
<td></td>
<td>DDTc</td>
<td>3.05 ± 0.15</td>
<td>2.035</td>
<td>2.00</td>
<td>43) Yamazaki et al., (1992)</td>
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<td>Terfenadine</td>
<td>11.82 ± 1.26</td>
<td>5.758</td>
<td>4.70</td>
<td>44) Ekins et al., (1999)</td>
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<td></td>
<td>Troreandomycin</td>
<td>3.59 ± 0.63</td>
<td>1.751</td>
<td>10.00</td>
<td>45) Zhou et al., (1993)</td>
<td></td>
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<td></td>
<td>ketoconazole</td>
<td>0.13 ± 0.04</td>
<td>0.061</td>
<td>0.11</td>
<td>46) Venkatakrishnan et al., (1998)</td>
<td></td>
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<td></td>
<td>erythromycin</td>
<td>162.61</td>
<td>79.22</td>
<td>20.00</td>
<td>47) Schmider et al., (1995)</td>
<td></td>
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<td></td>
<td>clarithromycin</td>
<td>&gt;100</td>
<td>97.44\textsuperscript{c)}</td>
<td>43.00</td>
<td>48) Zhao et al., (1999)</td>
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<td>48) Zhao et al., (1999)</td>
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from IC50 values at the MTP assay and was compared to the test compounds for each CYP isoform was calculated. The results for each CYP isoform, except for CYP2B6 and CYP3A4, are shown in Table 2. The IC50 values of a typical substrate (kininergic protein) was incubated with AMMC (0.75, 1.5, 3 and 6 μM) and recombinant CYP2D6 microsomes on a 96 well plate. The mean ± S.D. of triplicate determinations. The IC50 value of quinidine was practically determined using a secondary plot of Vmax versus inhibitor concentration from Lineweaver-Burk plots. We used 6 concentrations of the inhibitor and 4 concentrations of the substrate. The IC50 value was calculated from IC50 at the MTP assay based on the following equation; IC50 = (Km Vmax/S), where IC50 is inhibition constant calculated from IC50, Km and S are inhibition constant calculated from IC50, 50% inhibition percentage, affinity of AMMC on recombinant CYP2D6 and AMMC concentration used in this study, respectively. The IC50 value is represented as mean ± S.D. at each substrate concentration.

**The Ki-cal was observed by a secondary plot of Kd/Vmax, versus inhibitor concentration from Lineweaver-Burk plots.**

**The Ki-cal of quinidine was calculated from IC50 at the MTP assay based on the following equation; IC50 = (Km Vmax/S), where IC50 is inhibition constant calculated from IC50, Km and S are inhibition constant calculated from IC50, 50% inhibition percentage, affinity of AMMC on recombinant CYP2D6 and AMMC concentration used in this study, respectively. The IC50 value is represented as mean ± S.D. at each substrate concentration.**

**The Ki-cal of quinidine was calculated from IC50 at the MTP assay based on the following equation; IC50 = (Km Vmax/S), where IC50 is inhibition constant calculated from IC50, Km and S are inhibition constant calculated from IC50, 50% inhibition percentage, affinity of AMMC on recombinant CYP2D6 and AMMC concentration used in this study, respectively. The IC50 value is represented as mean ± S.D. at each substrate concentration.**

For quinidine, which is a competitive inhibitor, intersecting point of each linear curve was almost on the y-axis of Lineweaver-Burk plots. The IC50 values of typical substrates for each CYP isoform were approximately 162.6 μM. IC50 value obtained from the experiment at 1.5 μM AMMC was 0.0056 μM and the IC50 (0.024 μM) from the IC50 corresponded closely to the IC50 (0.0024 μM) from the IC50. Although there are many inhibition patterns among drug-drug interactions, we assumed that all inhibitions in this study are competitive. This assumption was made in order to readily estimate the Ki values of test compounds through the MTP assay as HTS at the stage of drug discovery. The Ki-cal of 26 test compounds for each CYP isoform was calculated from IC50 values at the MTP assay and was compared with the Ki-cal in HLM contained in much literature using conventional methods such as HPLC analysis.

**Figure 3** shows the relationship between these values for each CYP isoform, except for CYP2B6 and CYP2C8. With respect to each CYP isoform, there was a good correlation between Ki-cal values from recombinant CYP isoforms and the Ki-cal values from HLM. For other test compounds except lansoprazole, tranylcypromine (CYP2C19) and TAO (CYP3A4), the difference between the Ki-cal and the Ki-cal was less than approximately four-folds (Table 2).

As shown in **Figure 4**, even when all the inhibitory effects of the 26 test compounds on each CYP activity were compared on the same figure, a good correlation (r2 = 0.7306) was found between the Ki-cal and the Ki-cal. In addition, the correlation coefficient obtained from only the inhibitors (positive control) and that obtained from only typical substrates was r2 = 0.9964 (except data on tranylcypromine for CYP2C19) and r2 = 0.686, respectively. These findings show that the Ki values calculated from recombinant CYP isoforms obtained using the MTP assay reflects well the Ki values obtained using human liver microsomes.

**Estimation of time-dependent inhibition using the preincubation method:** Using the MTP assay with the preincubation method, we attempted to estimate the inhibitory pattern (competitive inhibition as substrate metabolism or mechanism based inhibition) by preincubation of CYP isoforms and test compound, with respect to five CYP isoforms that are mainly involved in the metabolism of most drugs on the market. Test compounds used were furafylline, α-naphthoflavone, sulfaphenazole, tranylcypromine, quinidine, ketoconazole and erythromycin, well known as selective inhibitors of CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4, respectively. Phenacetin (CYP1A2), diclofenac, phenytoin (CYP2C9), S-mephenytoin, lansoprazole (CYP2D6), dextromethorphan, bufuralol (CYP2D6), and terfenadine (CYP3A4) were also used for this estimation, as these are typical substrates for each CYP isoform.

**Figure 5** shows the preincubation time-dependent change in IC50 values of these compounds for each CYP isoform. The IC50 values of furafylline on recombinant CYP1A2 after preincubation for 0 min, 10 min and 30 min were 1.56 μM, 0.122 μM and 0.042 μM, respectively. The values for erythromycin on recombinant CYP3A4 after preincubation for 0 min, 10 min and 30 min were 162.6 μM, 4.389 μM and 1.959 μM, respectively. These mechanism based inhibitors, strongly inhibited each CYP activity, depending on the preincubation time. On the other hand, after preincubation for 10 or 30 min, the IC50 values of typical substrates (phenacetin, diclofenac, S-mephenytoin, dextromethorphan, bufuralol and terfenadine) for each CYP isoform were approximately 2.2, 4.3, 1.4, 1.8, 1.7 and 3.3 times larger than found in the case of co-incubation, respectively. The inhibitory effects of sulfaphenazole, tranylcypromine and ketoconazole slightly changed compared to the case of co-incubation, but the IC50 value of quinidine was not observed. The IC50 values of test compounds through the MTP assay were compared to the test compounds for each CYP isoform was calculated. The Ki-cal values from recombinant CYP2D6 microsomes. Quinidine (0–0.01 μM) was incubated with AMMC (0.75, 1.5, 3 and 6 μM) and recombinant CYP2D6 microsomes on a 96 well plate. Data are the mean ± S.D. of triplicate determinations. The Ki-cal value of quinidine was practically determined using a secondary plot of Kd/Vmax versus inhibitor concentration from Lineweaver-Burk plots. We used 6 concentrations of the inhibitor and 4 concentrations of the substrate. The Ki-cal of quinidine was calculated from IC50 at the MTP assay based on the following equation; IC50 = (Km Vmax/S), where IC50 is inhibition constant calculated from IC50, 50% inhibition percentage, affinity of AMMC on recombinant CYP2D6 and AMMC concentration used in this study, respectively. The IC50 value is represented as mean ± S.D. at each substrate concentration.

**Figure 2** shows the preincubation time-dependent change in IC50 values of these compounds for each CYP isoform. The IC50 values of furafylline on recombinant CYP1A2 after preincubation for 0 min, 10 min and 30 min were 1.56 μM, 0.122 μM and 0.042 μM, respectively. The values for erythromycin on recombinant CYP3A4 after preincubation for 0 min, 10 min and 30 min were 162.6 μM, 4.389 μM and 1.959 μM, respectively. These mechanism based inhibitors, strongly inhibited each CYP activity, depending on the preincubation time.
In this study, we examined the correlation between $K_i$ values estimated from MTP assay and the $K_i$ values obtained from HLM, regarding nine CYP isoforms mainly existing in the human liver. In addition, we advocated the methodology to be used to readily estimate the inhibitory patterns of drug candidates on CYP activity in order to predict metabolic properties for drug-drug interactions. First, for quinidine, a competitive inhibitor for CYP2D6, the $K_i$ values obtained from IC$_{50}$ of 51.7 $\mu$M, 0.101 $\mu$M and 63.67 $\mu$M, respectively. After preincubation for 10 min or 30 min, the IC$_{50}$ values were 4.8 $\mu$M, 0.406 $\mu$M and 82.12 $\mu$M, respectively.

**Discussion**

In this study, we examined the correlation between $K_i$ values estimated from MTP assay and the $K_i$ values obtained from HLM, regarding nine CYP isoforms mainly existing in the human liver. In addition, we advocated the methodology to be used to readily estimate the inhibitory patterns of drug candidates on CYP activity in order to predict metabolic properties for drug-drug interactions. First, for quinidine, a competitive inhibitor for CYP2D6, the $K_i$ values calculated from IC$_{50}$ and secondary plots of Lineweaver-Burk plots. The $K_i$ values of 26 compounds were compared with the $K_i$ obtained from HLM and a good correlation was found (Fig. 4). These findings are consistent with a report by Bapiro et al., and show that the MTP assay is a very useful HTS method to estimate $K_i$ values indicating the potency of the inhibitory effect of drug candidates on CYP activity in HLM at the stage of drug discovery. In addition, the assay is useful for decisions on experimental conditions to assess drug-drug interactions at the stage of drug development.

In this study, however, the correlation coefficient between the $K_i$ and the $K_i$ of substrates was slightly lower than that of selective inhibitors (Fig. 4). We considered that the inhibitory effect of certain substrates on HLM is smaller than that on recombinant CYPs because these substrates may be metabolized by other CYP isoforms in HLM, differently from the case of specific inhibitors. Moreover, the $K_i$ values of lanoprazole, tranylcypromine (for CYP2C19) and TAO from recombinant CYPs were much smaller than the $K_i$ value from HLM. Taavitsainen et al., and Zhang
TAO is a potent mechanism based inhibitor.\textsuperscript{17) Nomeir result in a slight difference in incubation time, because that the lower \( K_i \) values are important for prediction of CYP3A4 inhibition.\textsuperscript{16} For tranylcypromine, our finding agreed with the result reported by Zhang et al.\textsuperscript{16} The concentration of lansoprazole in HLM microsomes is likely to be due to the absence of other P450s present in HLM that may interact with the inhibitory effect of TAO on CYP3A4 activity strongly increased 1.5 and 3.3 times after preincubation for 10 min and 30 min, respectively. The increase in the \( IC_{50} \) values of phenacetin and terfenadine may result in a decrease in inhibitor concentration in the incubation medium, because these compounds are metabolized by CYP1A2 or CYP3A4 during preincubation. Results show that the inhibitory pattern of these compounds differs, and our findings support evidence that furafylline\textsuperscript{21,22} and erythromycin\textsuperscript{23,24} are mechanism based inhibitors for CYP1A2 and CYP3A4 and that phenacetin\textsuperscript{25} and terfenadine\textsuperscript{22} usually are used as typical substrates for these CYP isoforms.

Diclofenac, S-mephenytoin, dextromethorphan and bufuralol exhibited weak inhibition for CYP2C9, CYP2C19 and CYP2D6 activity, depending on the preincubation time. These results support evidence that diclofenac, S-mephenytoin, dextromethorphan and bufuralol are substrates of those CYP isoforms. In this study, the \( IC_{50} \) value of quinidine, a selective CYP2D6 inhibitor, did not change with preincubation. If quinidine were a substrate of CYP2D6, the \( IC_{50} \) values would change greatly with preincubation. Favreau et al.\textsuperscript{19} reported that the inhibition curve of quinidine did not shift depending on reaction time. In fact, quinidine competitively inhibits dextromethorphan O-demethylation and sparteine hydroxylation,\textsuperscript{25} but the compound is not metabolized by CYP2D6. These findings agree with the report by Nielsen et al.\textsuperscript{26} that quinidine is not a substrate for CYP2D6, but that it is metabolized by CYP3A4 in HLM.

Next, the inhibitory effect of compound A on CYP1A2, CYP2D6 and CYP3A4 activities after preincubation was evaluated using the same method. The
Fig. 5. Effect of preincubation on IC_{50} values of test compounds on five recombinant CYP isoforms. Preincubation time was 10 or 30 min. All data are from duplicate or triplicate determinations. The compounds used in this study are inhibitors, as positive controls, typical substrates and a new drug candidate.

IC_{50} values of compound A on CYP1A2 after preincubation were remarkably smaller than those found in case of the co-incubation. However, the IC_{50} values on CYP2D6 and CYP3A4 were 4 and 1.3 times larger than those in case of co-incubation. Therefore compound A might be a mechanism based inhibitor for CYP1A2 and a substrate for CYP2D6 and CYP3A4. Namely, the increase of IC_{50} values after preincubation suggests that the test compound is a substrate of the CYPs examined. On the other hand, the decrease in IC_{50} values after preincubation suggests that the test compound is a mechanism based inhibitor for the CYPs examined.

Consequently, we could readily estimate the inhibitory pattern of commercially available drugs on five CYP isoforms, using MTP assay with the preincubation method. In the second study, however, the conventional study using HLM is needed to identify the inhibition mechanism to predict the drug-drug interaction, because with the preincubation method using the MTP assay it is impossible to classify competitive and non-competitive inhibition.

After rapid development of combinatorial chemistry and the HTS for pharmacological efficacy, drug metabolism and pharmacokinetic studies have been regarded as the bottleneck at the drug discovery and development. Inhibition studies using the MTP assay have been done routinely and automatically as HTS method at the stage of drug discovery. However, regarding
evaluation of metabolic stability, even if the 96 well plate-formats and LC/MS/MS analysis were applied automatically for the assessment, the throughput is very low. For this reason, there is a time limitation in capacity for sample treatments with the LC/MS/MS analysis, because the parent drug has to be measured directly. Therefore, the methodology we described in this study might be utilized as the beneficial HTS to indirectly estimate CYP isoforms involved in the metabolism at stage of drug discovery.

In conclusion, our study indicates that the MTP assay is a useful HTS method to evaluate inhibitory effects of new drug candidates on 9 CYP isoforms in HLM in that the probe used at the assay is not radiometric and the throughput is considerably higher than that obtained with LC/MS/MS analysis. Moreover, the MTP assay with the preincubation method is applicable as a beneficial HTS to estimate inhibition patterns of new drug candidates on CYP isoforms and to readily estimate main CYP isoforms responsible for the metabolism. When this method is automated, throughput to evaluate drug metabolism and inhibition will increase.

References


