IN VITRO DRUG METABOLISM BY A HUMAN P450 EXPRESSION SYSTEM USING VACCINIA VIRUS.


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P450s are a superfamily of heme-containing monooxygenases and important in the metabolism of numerous physiological substrates and foreign compounds. It has been established that there are at least 30 distinct human isoforms of P450. Four families containing numerous individual P450s are mainly responsible for metabolizing foreign compounds. A cDNA expression system in which individual human P450s are synthesized in cultured human hepatoma (Hep G2) cells infected with a recombinant vaccinia virus containing human P450 cDNA has been constructed.

To select drugs that have more effect and less side-effect is an important in developing a new drug. Most of drugs are metabolized by a variety of enzymes, and these metabolic processes generally produce metabolites that are less toxic than the parent compound, in some cases the metabolites may be more reactive, producing toxic effects. The metabolic profiling of drugs undergoing development is, therefore, necessary to assess the effect and toxicity of the proposed drug. These metabolism studies are carried out in an in vivo study using experimental animals and in an in vitro study using liver from various animal species and humans. However, we are facing increased pressure to minimize the use of animals and ethical problem to use human liver. Furthermore, there are species
interactions as well as assessing their effects and the toxicity. It is also helpful in assessing whether or not drugs are in fact metabolized by these isoforms in relation to polymorphism.

P450s are a superfamily of heme-containing monooxygenases and important in the metabolism of numerous physiological substrates and foreign compounds. However, P450s alone are enzymatically inactive in the absence of an electron transport systems. Microsomal P450s use the flavoprotein, NADPH P450 reductase and, in some cases, cytochrome b₅ as a conduit to collect electrons from NADPH.

It has been established that there are at least 30 distinct human isoforms of P450 (Nelson et al., 1993). Four families containing numerous individual P450s are mainly responsible for metabolizing foreign compounds (Gonzalez, 1992). A cDNA expression system in which individual human P450s are synthesized in cultured human hepatoma (Hep G2) cells infected with a recombinant vaccinia virus containing human P450 cDNA has been constructed. The Hep G2 cells used for P450 expression contain much these electron transport enzymes, which cause the assay system much more reliable and useful. Several investigations using this system have been carried out to elucidate the identity of specific P450 isoforms which are involved in carcinogen and drug metabolism (Aoyama et al., 1990; Rettie et al., 1992; Flammang et al., 1992). These approaches can use to predict human P450 isoforms reflecting on drug metabolism and the profile of drug metabolism in humans.

**Experimental procedure**

Hep G2 cells were infected with a recombinant vaccinia virus containing the cDNA for one of the following; CYP1A2, 2A6, 2B6, 2C8, 2C9, 2D6, 2E1, 3A3, 3A4, and 3A5. Detailed information concerning
the construction of these recombinant viruses has been published elsewhere (Gonzalez et al., 1991). Control Hep G2 cells were infected with wild-type vaccinia virus. The cells were maintained for 20 hrs in Dulbecco's Modified Eagle's medium (high glucose) supplemented with 10 % fetal calf serum (FCS) at 37°C in 5 % CO₂ in air. For metabolism studies, the medium was exchanged for that without FCS and phenol red, containing various substrates. One to 24 hrs later, the metabolites in the medium and cells were analyzed. Within the period of metabolism, P450 expression levels in the vaccinia-infected cell lysates ranged from 10 to 20 pmol/mg protein. The level of NADPH P450 reductase in Hep G2 cells was 0.59 nmol/min/mg cell lysate protein, and the amount of cytochrome b₅ was 10.5 pmol/mg cell lysate protein.

Substrate specificity in human P450 isoforms (Table 1)

We studied the substrate specificity in ten human P450 isoforms expressed in Hep G2 cells using a recombinant vaccinia virus. Caffeine N3-demethylation was found to be suitable markers for the CYP1A2 isoform, and the CYP3A subfamily mediated testosterone 6β-hydroxylation and diazepam 3-hydroxylation. Other assays included coumarin 7-hydroxylation with CYP2A6, 7-benzylxoxyresorfin O-debenzylation and diazepam N-demethylation with CYP2B6, tolbutamide methyl-hydroxylation with CYP2C8 and 2C9, bufuralol 1′-hydroxylation and dextromethorphan O-demethylation with CYP2D6, and aniline 4-hydroxylation as well as chlorzoxazone 6-hydroxylation with CYP2E1. However, some of their substrates were metabolized by multiple P450 subfamilies at high concentrations. As catalysts of foreign compound metabolism, P450s are quite unique; a single P450 can oxidize a large number of different substrates. Some chemicals can also be metabolized at different positions by various P450 isoforms, although the rates of
<table>
<thead>
<tr>
<th>P450 isoform</th>
<th>Substrate</th>
<th>Reaction</th>
<th>Activity (µM / hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Caffeine</td>
<td>N3-Demethylation</td>
<td>5.97</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>Theophylline</td>
<td>8-Hydroxylation</td>
<td>1.65</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>Coumarin</td>
<td>7-Hydroxylation</td>
<td>4.17</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>7-Benzylxoxyresorfin</td>
<td>O-debenzylation</td>
<td>0.09</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>Diazepam</td>
<td>N-demethylation</td>
<td>4.44</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>Tolbutamide</td>
<td>Methyl-hydroxylation</td>
<td>0.05</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Tolbutamide</td>
<td>Methyl-hydroxylation</td>
<td>0.18</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Phenytoin</td>
<td>4-Hydroxylation</td>
<td>0.08</td>
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<tr>
<td>CYP2D6</td>
<td>Dextromethorphan</td>
<td>O-demethylation</td>
<td>0.39</td>
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<tr>
<td>CYP2D6</td>
<td>Bufuralol</td>
<td>1'-Hydroxylation</td>
<td>1.03</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>Aniline</td>
<td>4-Hydroxylation</td>
<td>9.33</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>Chlorzoxazone</td>
<td>6-Hydroxylation</td>
<td>10.46</td>
</tr>
<tr>
<td>CYP3A3</td>
<td>Testosterone</td>
<td>6β-Hydroxylation</td>
<td>15.1</td>
</tr>
<tr>
<td>CYP3A3</td>
<td>Diazepam</td>
<td>3-Hydroxylation</td>
<td>3.71</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Testosterone</td>
<td>6β-Hydroxylation</td>
<td>25.3</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Diazepam</td>
<td>3-Hydroxylation</td>
<td>5.27</td>
</tr>
<tr>
<td>CYP3A5</td>
<td>Testosterone</td>
<td>6β-Hydroxylation</td>
<td>8.50</td>
</tr>
<tr>
<td>CYP3A5</td>
<td>Diazepam</td>
<td>3-Hydroxylation</td>
<td>14.0</td>
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</table>

catalysis can vary, particularly at low substrate concentrations. At high concentrations of a chemical, more than one P450 may be involved in its metabolism. At low concentrations of a chemical, a single P450 isoform might predominate. These results suggest that the metabolic activity of P450 isoforms should be measured at low substrate concentrations so that their substrate specificity can be defined in human liver microsomes as well as in purified or cDNA-expressed human P450s.
Table 2
The contribution of demethylation and hydroxylation pathways to caffeine metabolism in different systems

<table>
<thead>
<tr>
<th>System</th>
<th>Primary metabolites formed (%)</th>
<th>Caffeine</th>
<th>TB</th>
<th>PX</th>
<th>TP</th>
<th>TMU</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo (Human)</td>
<td>Unknown</td>
<td></td>
<td>20</td>
<td>73</td>
<td>8</td>
<td>2</td>
<td>Fuhr et al., 1992</td>
</tr>
<tr>
<td>Human CYP1A2</td>
<td>0.5 mM</td>
<td></td>
<td>10</td>
<td>85</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human CYP1A2</td>
<td>10 mM</td>
<td></td>
<td>13</td>
<td>83</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human liver Microsomes</td>
<td>0.5 mM</td>
<td></td>
<td>10</td>
<td>66</td>
<td>9</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Human liver Microsomes</td>
<td>10 mM</td>
<td></td>
<td>9</td>
<td>32</td>
<td>8</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>Mouse CYP1A1</td>
<td>10 mM</td>
<td></td>
<td>10</td>
<td>84</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Mouse CYP1A2</td>
<td>10 mM</td>
<td></td>
<td>33</td>
<td>63</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

—: Below detection limit.

Table 3
Contribution of demethylation and hydroxylation pathways to theophylline metabolism in different systems

<table>
<thead>
<tr>
<th>System</th>
<th>Primary metabolites formed (%)</th>
<th>Theophylline</th>
<th>3X</th>
<th>1X</th>
<th>DMU</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo (Human)</td>
<td>Unknown</td>
<td></td>
<td>15</td>
<td>24</td>
<td>61</td>
<td>Fuhr et al., 1992</td>
</tr>
<tr>
<td>Human CYP1A2</td>
<td>1 mM</td>
<td></td>
<td>28</td>
<td>32</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Human CYP1A2</td>
<td>10 mM</td>
<td></td>
<td>21</td>
<td>23</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>Human liver Microsomes</td>
<td>1 mM</td>
<td></td>
<td>27</td>
<td>10</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>Human liver Microsomes</td>
<td>10 mM</td>
<td></td>
<td>15</td>
<td>12</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>Mouse CYP1A1</td>
<td>10 mM</td>
<td></td>
<td>10</td>
<td>29</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>Mouse CYP1A2</td>
<td>10 mM</td>
<td></td>
<td>2</td>
<td>16</td>
<td>83</td>
<td></td>
</tr>
</tbody>
</table>

Caffeine and theophylline metabolism by human P450 isoforms (Tables 2 and 3)

We investigated caffeine metabolism, which has been used as an index of CYP1A activity, using a vaccinia virus cDNA expression system in which cDNAs for ten human P450 isoforms, and mouse CYP1A1 and
1A2 were respectively expressed. Additionally, the ability of these P450s to metabolize theophylline was studied. The metabolism of caffeine and theophylline was also investigated using human liver microsomes. The demethylation of these drugs was both mediated by human 1A2, as well as by mouse 1A1 and 1A2, and at high caffeine concentration (10 mM), 2D6, 2E1, 3A3, and 3A4 were also involved. The 8-hydroxylation of theophylline was mediated by human 2E1 as well as by human 1A2, and mouse 1A1 and 1A2. Whereas, the 8-hydroxylation of caffeine was not mediated by any of human P450 isoforms tested, and only by mouse 1A1 and 1A2 at very low levels of activity. The major metabolites of caffeine and theophylline by P450 were paraxanthine and 1,3-dimethylurate, respectively. These results reflect the metabolism in humans (in vivo), whereas the predominant metabolic pathways of 0.5 and 10 mM caffeine in human liver microsomes were 3-demethylation and 8-hydroxylation, respectively. Furthermore, the metabolic profiles of caffeine and theophylline by human 1A2 were similar to those by mouse 1A1, but not by mouse 1A2. These suggest that human P450 expression system using vaccinia virus in this study, but not the system using human liver microsomes, will be especially applicable to predict the metabolic behaviour of drugs metabolized by P450 isoforms in humans.

**Diazepam metabolism by human P450 isoforms**

Diazepam is a minor tranquilizer that undergoes N-demethylation and 3-hydroxylation to form nordiazepam and temazepam, respectively, in the liver. Studies using human liver microsomes have suggested that the CYP2C and CYP3A subfamilies are involved in diazepam metabolism which is also correlated with that of S-mephenytoin. However, the contribution of these P450s to diazepam metabolism in humans remains
unclear. Among the tested isoforms, diazepam was significantly
demethylated by 2B6, 3A3, 3A4 and 3A5, with 2B6 exhibiting the highest
rate, as well as hydroxylated by the latter three, of which 3A5 was the
most active. Under our conditions, however, 2C8 and 2C9 did not
metabolize diazepam. Since genetic polymorphism occurs in the human
liver CYP2C subfamily, it is possible that other members of this group
participate in diazepam metabolism. However, this remains to be
elucidated. Our results suggest that in the human liver, the metabolism of
diazepam to nordiazepam is mediated by 2B6 and 3A subfamily and to
temazepam, by 3A subfamily.

**Drug metabolism in relation to CYP2D6 polymorphism**

In human, polymorphisms in drug metabolizing enzymes are
important in drug therapy. Some drugs exert their actions over a narrow
range of concentrations or therapeutic index. Thus, individuals lacking
an enzyme that inactivates a drug could experience hyperresponses or
toxicity due to high serum concentrations of the active drug. The
desbenzquine/sparteine drug oxidation polymorphism is due to mutant
alleles of the enzyme CYP2D6 (Gonzalez and Meyer, 1991). The
CYP2D6 polymorphism is associated with metabolism of over 30
clinically used drugs (Eichelbaum and Gross, 1990). Among these, we
studied bufuralol and dextromethorphan metabolism by human P450
isoforms expressed in Hep G2 cells using a recombinant vaccinia virus.
The 1'-hydroxylation of bufuralol was mediated not only by CYP2D6,
but also by CYP1A2. The Km values of the reaction were 3.97 and 64.3
μM, and those of the Vmax were 2.90 and 1.38 nmol/hr/plate of
CYP2D6 and CYP1A2, respectively. These results suggest that the
reaction is catalyzed by the CYP2D6 at a low substrate concentration, but
by both CYP2D6 and CYP1A2 at a high substrate concentration. As the
CYP1A2 content in human liver is fairly higher than the CYP2D6
content, the index of CYP2D6 using human liver microsomes should be
measured at a low bufuralol concentration. The O-demethylation of
dextromethorphan was mediated by CYP2C9 as well as by CYP2D6. The
Km values of the reaction were 2.20 and 149 \mu M, and those of the Vmax
were 1.60 and 2.99 nmol/hr/plate of CYP2D6 and CYP2C9, respectively.
Furthermore, the level of CYP2C9 in the human liver is also higher than
that of CYP2D6. These suggest that, at a low concentration of
dextromethorphan, CYP2D6 mainly mediates the O-demethylation,
whereas at a high concentration, CYP2C9 predominates.

**Chlorzoxazone metabolism by human P450 isoforms**

Chlorzoxazone has been suggested as an in vivo probe for CYP2E1
(Peter et al., 1990). However, population studies of this muscle-relaxing
drug have not yet been carried out. We studied the specificity of
chlorzoxazone using vaccinia virus expressed human P450 isoforms. The
6-hydroxylation of chlorzoxazone was mediated by CYP1A2 as well as by
CYP2E1. The Km values of CYP1A2 and CYP2E1 for the reaction were
5.69 and 232 \mu M, respectively. However, the Vmax value of CYP2E1
for the reaction was about 8.3-fold higher than that of CYP1A2. These
results raise questions about the suitability of chlorzoxazone as an in vivo
prove of hepatic CYP2E1 activity. Furthermore, in hepatic
microsomes, the Km (40 \mu M) was different from that of either CYP1A2
and CYP2E1. This is explained by the fact that the expression levels of
CYP1A2 and CYP2E1 in the human liver are similar, and both P450s
catalyze the 6-hydroxylation of chlorzoxazone. Furthermore, it is
suggested that the role of CYP2E1 in 6-hydroxychlorzoxazone formation
at the physiological chlorzoxazone concentration of 30-60 μM is similar to that of CYP1A2.

**Mexazolam metabolism by human P450 isoforms**

Mexazolam, a benzodiazepinoxazole-anxiolytic, was first N-dealkylated to 7-chloro-5-(2-chlorophenyl)-1-hydro-3-dihydro-2H-1,4-benzodiazepin-2-one (M1), then hydroxylated to lorazepam in rat liver microsomes (Ikeda et al., 1998). Studies using rat liver microsomes have suggested that each metabolic step is mediated by the P450 induced by phenobarbital. However, the P450 isoform(s) contributing to mexazolam metabolism remains unclear. Mexazolam was not metabolized in Hep G2 cells infected with wild type vaccinia virus. In Hep G2 cells expressing CYP2B6, 3A3, 3A4, or 3A5, mexazolam was metabolized to M1, and furthermore, to lorazepam by CYP3A subfamily. However, mexazolam was not metabolized by CYP1A2, 2A6, 2C8, 2C9, 2D6 and 2E1. Furthermore, the chemical structure of the major metabolite of mexazolam by cDNA-expressed human CYP3A4 was analyzed by mass spectrometry, which confirmed that it was M1.

**Conclusion**

The increasing availability of cDNA-expressed P450s, will allow predictive tests to determine how drugs or other chemicals are metabolized by individual P450 isoform(s) before being administered to humans. If a drug is processed to toxic metabolites, or by polymorphic P450s, it might allow some structural modification of the parent compound. P450s have been produced using vaccinia virus, retrovirus, baculovirus, COS cells, V79 cells, yeast and bacteria. P450 system using vaccinia virus in the present study constantly expresses high level of
individual P450 isoforms. P450 system used here has a high level of the 
P450 holoenzyme, and the host cells contain sufficient amounts of 
mammalian NADPH P450 reductase and cytochrome b5. It means that our 
system expresses higher contents of P450 and has higher turnover rate for 
drugs than other system. This suggests that our system can accurately 
mirror the metabolic profile of drugs and, furthermore, can identify 
which P450 isoforms are involved in metabolism of a particular drug. 
This system will be especially applicable to drugs metabolized by human 
P450 isoforms in relation to polymorphisms. Furthermore, this system 
will be able to produce tailor-made metabolites.

We need to derive a more knowledge of the structure-activities 
relating to substrate specificity for drug metabolism by various human 
P450 isoforms. If possible, we should build up a chemical database about 
drug metabolism. Therefore, we are necessary to study the metabolism 
of more compounds using cDNA-expressed human P450s. Furthermore, 
this strategy is applicable to develop the co-expression of human drug 
metabolizing enzymes, allowing the prediction of complete metabolic 
pathways in humans.

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