ROLES OF CYTOCHROMES P450 1A2, 2A6, AND 2C8 IN 5-FLUOROURACIL FORMATION FROM TEGAFUR, AN ANTICANCER PRODRUG, IN HUMAN LIVER MICROSONES

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ABSTRACT:

Tegafur, an anticancer prodrug, is bioactivated to 5-fluorouracil (5-FU) mainly by cytochrome P450 (P450) enzymes. The conversion from tegafur into 5-FU catalyzed by human liver microsomal P450 enzymes was investigated. In fourteen cDNA-expressed human P450 enzymes having measurable activities, CYP1A2, CYP2A6, CYP2E1, and CYP3A5 were highly active in catalyzing 5-FU formation at a tegafur concentration of 100 μM. Kinetic analysis revealed that CYP1A2 had the highest V_{max}/K_{m} value and that the V_{max} value of CYP2A6 was high in 5-FU formation. In human liver microsomes, the activities of 5-FU formation from 10 μM, 100 μM, and 1 mM tegafur were significantly correlated with both coumarin 7-hydroxylation (r = 0.83, 0.86, and 0.74) and paclitaxel 6a-hydroxylation (r = 0.77, 0.62, and 0.85) activities, respectively. Coumarin efficiently inhibited the 5-FU formation activities from 100 μM and 1 mM tegafur catalyzed by human liver microsomes that had high coumarin 7-hydroxylation activity. On the other hand, furafylline, fluvoxamine, and quercetin, as well as coumarin, showed inhibitory effects in liver microsomes that had high catalytic activities of 5-FU formation. The other P450 inhibitors examined showed weak or no inhibition in human liver microsomes. Polyclonal anti-CYP1A2 antibody, monoclonal anti-CYP2A6, and anti-CYP2C8 antibodies inhibited 5-FU formation activities to different extents in those two microsomal samples. These results suggest that CYP1A2, CYP2A6, and CYP2C8 have important roles in human liver microsomal 5-FU formation and that the involvement of these three P450 forms differs among individual humans.

Cytochrome P450 (P450) comprises a superfamily of enzymes involved in the oxidation of a great number of exogenous and endogenous compounds (Guengerich, 1995). Especially P450 enzymes in the CYP1, CYP2, and CYP3 families can play important roles not only in the metabolism of a variety of drugs but also in the metabolic activation of mutagens or in prodrug activation (Bertz and Granneman, 1997). There are large interindividual variations in the contents of P450 enzymes having measurable activities, CYP1A2, CYP2A6, CYP2C8 and CYP3A5 were highly active in catalyzing 5-FU formation. In human liver microsomes, the activities of 5-FU formation from 10 μM, 100 μM, and 1 mM tegafur were significantly correlated with both coumarin 7-hydroxylation (r = 0.83, 0.86, and 0.74) and paclitaxel 6a-hydroxylation (r = 0.77, 0.62, and 0.85) activities, respectively. Coumarin efficiently inhibited the 5-FU formation activities from 100 μM and 1 mM tegafur catalyzed by human liver microsomes that had high coumarin 7-hydroxylation activity. On the other hand, furafylline, fluvoxamine, and quercetin, as well as coumarin, showed inhibitory effects in liver microsomes that had high catalytic activities of 5-FU formation. The other P450 inhibitors examined showed weak or no inhibition in human liver microsomes. Polyclonal anti-CYP1A2 antibody, monoclonal anti-CYP2A6, and anti-CYP2C8 antibodies inhibited 5-FU formation activities to different extents in those two microsomal samples. These results suggest that CYP1A2, CYP2A6, and CYP2C8 have important roles in human liver microsomal 5-FU formation and that the involvement of these three P450 forms differs among individual humans.

1 Abbreviations used are: 5-FU, 5-fluorouracil; P450, general term for cytochrome P450; CYP, individual forms of P450.

Sayed and Sadee, 1982, 1983). Activation of tegafur to 5-FU can be performed in a reconstituted system containing purified rabbit CYP1A2 or CYP2B4 (Kawata et al., 1984). It has been reported that these reactions required NADPH and are inhibited by carbon monoxide and metyrapone (Kawata et al., 1984, 1987). From these findings, the formation of 5-FU from tegafur in the microsomal fraction is thought to be mediated by P450 enzymes, but there is no information concerning the mammalian P450 enzymes involved in 5-FU formation. A drug interaction of tegafur with anticancer drugs or phenytoin was suggested (Ogawa et al., 1988; Wakisaka et al., 1990), but the mechanism is not clear yet. Concerning humans, a meeting abstract has suggested that CYP2A6 is involved in the conversion of tegafur into 5-FU (Ikeda et al., 1996). However, the roles of human P450s other than CYP2A6 were not examined in detail.

In this study, the roles of human P450 enzymes involved in 5-FU formation from tegafur mainly at a substrate concentration of 100 μM, based on the clinical plasma concentration, were investigated with...
recombinant P450 enzymes and human liver microsomes. We report here that CYP1A2 and CYP2C8 as well as CYP2A6 have different contributions to the bioactivation of tegafur into 5-FU in individual human liver microsomes.

Materials and Methods

Chemicals. 5-FU and 5-bromouracil were obtained from Wako Pure Chemicals (Osaka, Japan) and Sigma (St. Louis, MO), respectively. Tegafur (>99.9%) and its oxidative metabolites (shown in Fig. 2A) were provided by Drs. Sekio Nagayama and Yasuro Kawaguchi, Taiho Pharmaceutical, Tokyo, Japan. Other chemicals used in this study were obtained from sources described previously or were of the highest qualities commercially available (Nakajima et al., 1999; Yamazaki et al., 1999).

Enzyme Preparations. Human liver microsomal sample HL-9 was prepared in 10 mM Tris-HCl buffer (pH 7.4) containing 0.10 mM EDTA and 20% (v/v) glycerol as described previously (Yamazaki et al., 1999). It corresponds to HL-134 (Guengerich, 1995) and HL-C18 (Shimada et al., 1999). The other human liver microsomes (HG3, -6, -23, -30, -42, -43, -56, -66, -70, -89, -93, and -112) were obtained from Gentest (Woburn, MA). The immunochemically determined P450 contents (except for CYP2C8 and CYP2C19) and specific catalytic activities of each P450 form in these microsomes were provided in the data sheets by the manufacturer; anti-CYP2C8 antibodies were obtained from Daiichi Pure Chemicals (Tokyo, Japan), and anti-rat CYP2A1 antibody was provided by Drs. Yoshihiko Funae and Susumu Imaoka, Osaka City University Medical School, Osaka, Japan. Monoclonal anti-human CYP2A6, anti-human CYP2C8, and anti-human CYP2E1 antibodies were also obtained from Gentest. The specificities of monoclonal antibodies were provided in the data sheets by the manufacturer; anti-CYP2C8 antibody is known to have no inhibitory effects on CYP2C9- or CYP2C19-mediated drug oxidations, and anti-CYP2A6 and anti-CYP2E1 antibodies have no effects on other forms.

Enzyme Assays. 5-FU formation activities were determined according to methods described elsewhere (El Sayed and Sadee, 1983; Loos et al., 1999) with slight modifications. The standard incubation mixture (final volume of 0.25 ml) contained human liver microsomes (0.5 mg of protein/ml); 100 mM potassium phosphate buffer (pH 7.4); an NADPH-generating system consisting of 0.5 mM NADP+, 5 mM glucose 6-phosphate, and 0.5 unit of glucose-6-phosphate dehydrogenase/ml; and tegafur (0.01–1 mM). In some cases, microsomes containing recombinant P450 enzymes (0.020 μM coexpressing P450 reductase were used. P450 inhibitors were dissolved in H2O (diethyldithiocarbamate and fluvoxamine), in CH3OH (coumarin, quercetin, sulfaphenazole, quinidine, p-nitrophenol, and ketoconazole), or in dimethylsulfoxide (α-naphthoflavone, furafylline, and paclitaxel). The final concentration of organic solvent in the incubation mixture was <1.0%. Incubations were carried out at 37°C for 30 min and terminated by adding 1.5 ml of 2 M NaCl/2 M HCl. 5-Bromouracil (2 μM) was added as an internal standard. The reaction mixture was extracted twice with ethyl acetate. After centrifugation at 900g for 10 min, the organic phase was evaporated to dryness under a gentle N2 stream. The residue was dissolved in a mixture of with baculovirus containing human P450 and human NADPH-P450 reductase cDNA inserts (Supersomes) were also obtained from Gentest. Insect microsomes expressing only NADPH-P450 reductase and cytochrome b5 were also used. Polyclonal anti-rat CYP1A2 and anti-rat CYP2C13 immunoglobulin G fraction were obtained from Daiichi Pure Chemicals (Tokyo, Japan), and anti-rat CYP2A1 antibody was provided by Drs. Yoshihiko Funae and Susumu Imaoka, Osaka City University Medical School, Osaka, Japan. Monoclonal anti-human CYP2A6, anti-human CYP2C8, and anti-human CYP2E1 antibodies were also obtained from Gentest. The specificities of monoclonal antibodies were provided in the data sheets by the manufacturer; anti-CYP2C8 antibody is known to have no inhibitory effects on CYP2C9- or CYP2C19-mediated drug oxidations, and anti-CYP2A6 and anti-CYP2E1 antibodies have no effects on other forms.

Figure 2. Representative HPLC chromatograms of tegafur metabolites catalyzed by recombinant P450 enzymes and human liver microsomes.
substrate concentrations of 10 μM, 100 μM, and 1 mM were used to determine which P450 forms are active in catalyzing the formation of 5-FU from tegafur at 1 mM concentrations. Insect microsomes expressing only NADPH-P450 reductase and cytochrome b₅ were used as control. Results are presented as means of duplicate determinations.

Tegafur was incubated at 37°C for 30 min with recombinant P450 enzymes (0.020 μM P450, Supersomes; Gentest) coexpressing NADPH-P450 reductase in the presence of an NADPH-generating system. Insect microsomes expressing only NADPH-P450 reductase and cytochrome b₅ were used as control. Results are presented as means of duplicate determinations.

Activities of 5-FU Formation Catalyzed by Recombinant P450 Enzymes Expressed in Baculovirus Systems. In our preliminary experiments with recombinant CYP2A6, the activities of 5-FU formation at 1 mM tegafur increased linearly for up to 40 min of incubation time and to 40 pmol of P450 per ml. Fourteen forms of P450 were used to determine which P450 forms are active in catalyzing the formation of 5-FU at substrate concentrations of 10 μM, 100 μM, and 1 mM (Fig. 3). CYP1A1, CYP1A2, CYP1B1, CYP2B6, and CYP3A5 showed high activities of 5-FU formation from 10 μM tegafur (Fig. 3A). At 100 μM tegafur, CYP2A6 and CYP2E1 were highly active in addition to CYP1A2 and CYP3A5 (Fig. 3B). In the 5-FU formation from 1 mM tegafur, CYP1A2 and CYP2A6 had the highest catalytic activities (Fig. 3C).

Kinetic analysis of 5-FU formation by recombinant P450 enzymes showed that CYP1A2 and CYP2A6 had Kₘ values of 0.36 and 1.10 mM and Vₘₐₓ values of 3.58 and 6.77 nmol/min/nmol P450, respectively (Table 1). The rates of 5-FU formation catalyzed by CYP2C8, CYP2C9, CYP2C19, and CYP2E1 were increased linearly up to concentrations of 2 mM tegafur; the apparent Kₘ values of these P450 forms were calculated to be >2 mM. The apparent Vₘₐₓ/Kₘ value of CYP1A2 was the highest among the six P450 forms tested (CYP2C8, 1.3; CYP2C9, 1.4; CYP2C19, 1.4; and CYP2E1, 3.2 ml/min/nmol P450).

Characterization of 5-FU Formation in Human Liver Microsomes. The rates of 5-FU formation from tegafur in standard reaction mixtures containing human liver microsomes (a sample of HL-9) were increased linearly with microsomal protein concentrations up to 1.0 mg/ml and with time up to 40 min (Fig. 4, A and B). 5-FU formation activities were increased in a substrate concentration-dependent manner (Fig. 4C).

Kinetic parameters for 5-FU formation from tegafur by recombinant P450 enzymes

<table>
<thead>
<tr>
<th>P450 Enzymes</th>
<th>Kₘ</th>
<th>Vₘₐₓ</th>
<th>Vₘₐₓ/Kₘ</th>
</tr>
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<tbody>
<tr>
<td>CYP1A2</td>
<td>0.36 ± 0.04</td>
<td>3.58 ± 0.11</td>
<td>9.9</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>1.10 ± 0.26</td>
<td>6.77 ± 0.78</td>
<td>6.2</td>
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</table>

*TABLE 1* Kinetic parameters for 5-FU formation from tegafur by recombinant P450 enzymes

Kinetic analyses for 5-FU formation from tegafur were determined at a P450 concentration of 0.020 μM (Supersomes, Gentest) and substrate concentrations between 25 μM and 2 mM. The kinetic parameters were calculated from the fitted curves using the computer program KaleidaGraph.
human liver microsomal sample HG3 (0.95 mM) was similar to that of recombinant CYP2A6 (1.10 mM) as shown in Table 1.

To investigate the interindividual differences in 5-FU formation activities, tegafur was incubated with 12 samples of human liver microsomes at substrate concentrations of 10 μM, 100 μM, and 1 mM (Fig. 5). All human liver samples had some catalytic activities of 5-FU formation. Interindividual differences in 5-FU formation activities from 1 mM tegafur (Fig. 5C) showed different patterns from those of 1 and 10 μM (Fig. 5, A and B).

Correlations between specific catalytic activities or the contents of each P450 form and rates of 5-FU formation from tegafur (10 μM, 100 μM, and 1 mM) were examined using human liver microsomes. Table 3 shows the relationships between 5-FU formation activities and phenacetin 0-deethylation, coumarin 7-hydroxylation, and paclitaxel 6α-hydroxylation activities. 5-FU formation activities did not correlate with phenacetin 0-deethylation activities at any substrate concentrations examined. 5-FU formation activities at 10 μM, 100 μM, and 1 mM tegafur were significantly correlated with the activities of coumarin 7-hydroxylation (r = 0.83, 0.86, and 0.74) and paclitaxel 6α-hydroxylation (r = 0.77, 0.62, and 0.85), respectively. Correlation coefficients (r) between rates of coumarin 7-hydroxylation and 5-FU formation activities at 10 and 100 μM tegafur were larger than the rate at 1 mM. On the other hand, the correlation coefficient (r) between paclitaxel 6α-hydroxylation and 5-FU formation activities at 1 mM tegafur was larger than the rates at 10 and 100 μM. The CYP2A6 contents were also significantly correlated with the 5-FU formation activities at 10 μM, 100 μM, and 1 mM tegafur (r = 0.80, 0.89, and 0.59, respectively). There was a significant correlation (r = 0.61) between the total P450 contents and 5-FU formation activities at 1 mM tegafur; however, no other catalytic activities or contents of individual P450 forms correlated with the 5-FU formation activities.

Effects of P450 Inhibitors and Anti-P450 Antibodies on 5-FU Formation Activities in Different Human Liver Microsomes. The effects of P450 inhibitors on the 5-FU formation activities catalyzed...
by liver microsomes of HG3 and HG30 were determined at a substrate concentration of 100 μM tegafur (Fig. 6, A and C). Coumarin (10 μM), a typical substrate of CYP2A6 (Yun et al., 1991), efficiently inhibited the 5-FU formation activities catalyzed by sample HG3 and was followed by fluvoxamine, an inhibitor of CYP1A2 (Jeppesen et al., 1996). On the other hand, fluvoxamine showed strong inhibition on the 5-FU formation activities by sample HG30. Furafylline, an inhibitor of CYP1A2 (Tassaneeyakul et al., 1994), and quercetin, an inhibitor of CYP2C8 (Rahman et al., 1994), showed inhibitory effects by ~50%, which was similar to that of coumarin (10 μM).

The effects of P450 inhibitors on the 5-FU formation activities were precisely investigated at a substrate concentration of 1 mM tegafur (Fig. 6, B and D). Only coumarin and fluvoxamine efficiently inhibited the microsomal 5-FU formation activities of sample HG3. However, in addition to coumarin and fluvoxamine, furafylline and quercetin had inhibitory effects on the 5-FU formation activities catalyzed by sample HG30. Sulfaphenazole, quinidine, diethyldithiocarbamate, p-nitrophenol, and ketoconazole showed weak or no inhibition in either human liver microsomal samples. In separate experiments, fluvoxamine (10 μM) also inhibited the 5-FU formation activities at 1 mM tegafur in recombinant CYP2C8 and CYP2C19 systems by 39 and 38%, respectively, but CYP2C9 was not inhibited in these assay conditions.
Polyclonal anti-CYP2A1 and monoclonal anti-CYP2A6 antibodies strongly inhibited the 5-FU formation activities by human liver microsomal sample HG3 at 100 µM tegafur (Table 4). On the other hand, the inhibitory effects of monoclonal anti-CYP2C8 and polyclonal anti-CYP2C13 antibodies were similar as those of anti-CYP2A antibodies in sample HG30. Anti-CYP1A2 antibodies showed weak inhibition on the 5-FU formation activities catalyzed by sample HG30. Anti-CYP2E1 antibodies did not inhibit these activities in the two human liver microsomal samples.

**Discussion**

P450-based cancer gene therapy is a novel prodrug activation strategy for cancer treatment. The identification of prodrug-activating enzymes is important for cancer chemotherapeutics to improve the safety and efficacy of prodrugs. A strategy of transduction of activating enzyme genes has been demonstrated for cyclophosphamide and ifosfamide, which are activated mainly by CYP2B enzymes to alkylating agents (Waxman et al., 1999). Tegafur is also one of the useful prodrugs administered orally as an anticancer agent and has been clinically used for ~20 years. Liver microsomal P450 enzymes have been reported as activating enzymes of tegafur in animals (El Sayed and Sadee, 1982). However, the roles of P450 forms are not yet well understood (Kawata et al., 1984), with only a meeting abstract suggesting a contribution of human CYP2A6 (Ikeda et al., 1996).

The maximum blood concentration of tegafur has been reported to be ~60 µM after 300 mg of tegafur and ~120 ± 72 µM (mean ± S.E., n = 20) after 750 mg of tegafur administered orally (Ogawa et al., 1988; Arima et al., 1989; Kanamitsu et al., 2000) and steady-state concentrations are in the range of ~50 to 180 µM (Kanamitsu et al., 2000). Accordingly, a substrate concentration of 100 µM tegafur was used mainly in this study.

Using different human liver samples containing varying levels of individual P450 enzymes and recombinant human P450 enzymes expressed in a baculovirus system, we obtained several pieces of evidence to support the view that different human P450 enzymes, particularly CYP1A2, CYP2A6, and CYP2C8, contribute to the 5-FU formation from tegafur in humans and that the roles of these P450 enzymes vary according to the human samples used. The results obtained in this study can be summarized as follows.

First, CYP1A2 is involved in the 5-FU formation from tegafur, especially at a low substrate concentration. Although the 5-FU formation activities did not correlate with the phenacetin O-deethylation activities or CYP1A2 contents in human liver microsomes, recombinant CYP1A2 had an apparently high $V_{max}/K_m$ value for 5-FU formation, and anti-CYP1A2 antibody showed inhibitory effects in the microsomal sample showing high 5-FU formation activity. In the experiments with P450 inhibitors, furafylline and fluvoxamine efficiently inhibited 5-FU formation activities from 100 µM tegafur catalyzed by the same sample. Fluvoxamine (50 and 10 µM) inhibited CYP1A2-mediated estradiol 2-hydroxylation and CYP2C19-mediated 5-mephenytoin 4′-hydroxylation activities to ~20 and ~30% at substrate concentrations of 100 and 200 µM in human liver microsomes, respectively (Yamazaki et al., 1997, 1998). However, in this study, the roles of CYP2C19 in microsomal 5-FU formation were thought to be minor because recombinant CYP2C19 showed moderate activities and the effects of monoclonal anti-CYP2C8 and polyclonal anti-CYP2C antibodies were not different. Fluvoxamine inhibited the 5-FU formation activities in recombinant CYP2C8 and CYP2C19. From these results, the effect of fluvoxamine on 5-FU formation activities in human liver microsomes appeared to be mainly the inhibition of CYP1A2 and partly that of CYP2C8. Further studies will be necessary using monoclonal anti-CYP2C9 or anti-CYP2C19 antibodies.

Second, paclitaxel 6a-hydroxylation activities were correlated with 5-FU formation activities, and the correlation coefficient (r) was high at 1 mM tegafur. Quercetin and anti-CYP2C8 antibody inhibited the 5-FU formation activities in the two microsomal samples to different extents. These data suggest the contribution of CYP2C8 in the activation of tegafur into 5-FU.

Finally, it was confirmed that CYP2A6 was also an important enzyme involved in 5-FU formation because recombinant CYP2A6 was highly active at concentrations of 100 µM and 1 mM tegafur. Coumarin 7-hydroxylation activities and CYP2A6 contents were significantly correlated with the rates of 5-FU formation in human liver microsomes. Moreover, coumarin and anti-CYP2A6 antibody inhibited effectively the 5-FU formation activities in human liver microsomes. The apparent $K_m$ value of recombinant CYP2A6 was similar to that of a human liver microsomal sample (HG3).

In our kinetic analysis, we could not detect biphasic parameters for 5-FU formation catalyzed by liver microsomes prepared from different human samples. Although it is not known at present why our present results showed monophasic parameters for 5-FU formation by human liver microsomes, it should be mentioned that the apparent $K_m$ values differed <2-fold in individual samples (Table 2).

It has been shown that there are interindividual variations in the levels of individual P450 enzymes in humans (Shimada et al., 1994). For example, CYP1A2 is induced in human livers by several chemicals such as polycyclic aromatic hydrocarbons, arylamines, and chlorinated hydrocarbons through the ingestion of charcoal-brioled foods, smoking, and other exposures (Guengerich, 1989; Guengerich and Shimada, 1991; Wrighton and Stevens, 1992). Whether there is genetic polymorphism of CYP2C8 is not known, but CYP2C8 has catalytic activities of paclitaxel 6a-hydroxylation, with 23-fold interindividual variation in human liver microsomes (Sonnichsen et al., 1995). CYP2A6 is reported to show genetic polymorphism, including gene deletion (Nunoya et al., 1999; Nakajima et al., 2000). These results support the idea that the contribution of P450 enzymes in the 5-FU formation from tegafur in human livers may be altered by using different human samples in which compositions of the various P450 enzymes differ. Different roles of the human P450 enzymes in individual human liver microsomes were also observed in drug oxidations such as azelastine N-demethylation (Nakajima et al., 1999), troglitazone oxidation (Yamazaki et al., 1999), and estrogen hydroxylations (Yamazaki et al., 1998).

Recently, tegafur has been used in combination with modulators for dihydropyrimidine dehydrogenase and orotate phosphoribosyltrans-
ferase such as uracil or gimestat and potassium ostata, respectively, to control the anticancer effects and side effects. It has been considered that tegafur is also activated in part into 5-FU by thymidine phosphorlase in the 100,000 g supernatant (cytosol) fraction (Sugata et al., 1986) and that 5-FU formed from tegafur is inactivated by dihydro-pyrimidine dehydrogenase (Katona et al., 1998). Therefore, further studies about 5-FU formation and inactivation with microsomal and cytosolic protein are required.

In conclusion, we showed that the participation of human CYP2A6, CYP1A2, and CYP2C8 in the bioactivation of tegafur differs among individual humans. These findings may be useful for the prediction of the drug interactions or pharmacokinetics of tegafur in vivo toward improving cancer therapy.

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References


