Human CYP1B1 and Anticancer Agent Metabolism: Mechanism for Tumor-Specific Drug Inactivation?

BERTRAN ROCHAT, JANINE M. MORSMAN, GRAEME I. MURRAY, WILLIAM D. Figg, and HOWARD L. MCLEOD
Departments of Medicine and Therapeutics (B.R., J.M.M., H.L.M.) and Pathology (G.I.M.), University of Aberdeen, Aberdeen, Scotland; and Medicine Branch, Division of Clinical Sciences, National Cancer Institute, National Institutes of Health, Bethesda, Maryland (W.D.F.)
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ABSTRACT
The cytochrome P450 1B1 (CYP1B1) is involved in the metabolism of procarcinogens and xenobiotics. Human CYP1B1 protein has been detected in a variety of tumors but is not detected in adjacent normal tissues or in liver. This suggests that CYP1B1 could biotransform anticancer agents specifically in the target cells. The interaction between CYP1B1 and 12 commonly used anticancer drugs was screened using an ethoxyresorufin deethylase assay. Four agents were competitive inhibitors of CYP1B1 activity: flutamide ($K_i = 1.0 \, \mu M$), paclitaxel ($K_i = 31.6 \, \mu M$), mitoxantrone ($K_i = 11.6 \, \mu M$), and doxorubicin ($K_i = 28.0 \, \mu M$). Doxorubicin ($K_i = 2.6 \, \mu M$) and daunomycin ($K_i = 2.1 \, \mu M$) were mixed inhibitors, while tamoxifen was a noncompetitive inhibitor ($K_i = 5.0 \, \mu M$). Vinblastine, vincristine, 5-fluorouracil, etoposide, and cyclophosphamide did not inhibit CYP1B1 activity. In vitro incubations with flutamide and CYP1B1 produced a metabolite consistent with 2-hydroxyflutamide. Comparison of kinetic parameters ($K_m$, $K_i$, $V_{max}$) for flutamide 2-hydroxylation by CYP1B1, CYP1A1, and CYP1A2 indicate that CYP1B1 could play a major role for flutamide biotransformation in tumors. The results obtained indicate that several anticancer agents inhibit CYP1B1 activity. Drug inactivation by CYP1B1 may represent a novel mechanism of resistance, influencing the clinical outcome of chemotherapy.

Recent reports have underlined the role of cytochromes P450 (CYPs) in the biotransformation of anticancer agents and in modulating cytotoxicity (Iyer and Ratain, 1998; McLeod, 1998). Because CYPs are mainly expressed in the liver, studies have focused on the role and activity of hepatic CYP isoenzymes. Unlike most CYPs, CYP1B1 has not been detected in human liver but appears to have significant protein expression in human tumors (Murray et al., 1997; McFadyen et al., 1999). Immunohistochemistry demonstrated CYP1B1 protein in a wide range of human cancers including breast, colon, lung, esophagus, skin, lymph node, brain, and testis, but no detectable CYP1B1 protein was observed in the normal tissues adjacent to the tumors (Murray et al., 1997; McFadyen et al., 1999).

CYP1B1 has been identified to biotransform various xenobiotics, such as ethoxyresorufin, theophylline, and caffeine (Shimada et al., 1997), and shows overlapping catalytic activities with CYP1A1 and CYP1A2 (Crespi et al., 1997; Shimada et al., 1997). CYP1B1 appears to also have an important role in the activation of environmental procarcinogens (Shimada et al., 1996; Crofts et al., 1997). CYP1B1 also biotransforms $17\beta$-estradiol to its 4-hydroxy-metabolites (Hayes et al., 1996). The rate of these pathways strongly suggests that CYP1B1 activity participates in endocrine regulation and toxicity of estrogens in vivo (Spink et al., 1998).

CYP1B1 may represent a mechanism for tumor-specific drug metabolism, and approaches are now underway to identify prodrugs that undergo activation by CYP1B1. However, this enzyme may also represent a mechanism for tumor-selective drug inactivation and subsequent resistance. We screened the capability of commonly used anticancer drugs to interact with CYP1B1 by focusing on 12 agents known to be metabolized by CYPs. Seven of the 12 agents tested inhibited CYP1B1 activity, including four competitive inhibitors. For the anticancer agents that inhibited CYP1B1 activity, the constants of inhibition ($K_i$) were determined and were consistent with achievable in vivo concentrations.

Further in vitro evaluations with flutamide observed the production of 2-hydroxyflutamide by CYP1B1, CYP1A1, and CYP1A2. Kinetic parameters of flutamide 2-hydroxylation indicate that CYP1B1 could play a major role in the biotransformation of flutamide. In conclusion, screening of 12 anticancer agents demonstrated data consistent with CYP1B1 substrates in that seven inhibit EROD activity mediated by CYP1B1 in the low micromolar range. This suggests that CYP1B1 could play an important role for the pharmacological efficiency in the cancer cells.

ABBREVIATIONS: CYP, cytochrome P450; EROD, ethoxyresorufin-O-deethylase; FM, flutamide metabolite; HPLC, high performance liquid chromatography.
Experimental Procedures

Materials. Docetaxel was a gift from Rhône-Poulenc-Rorer (Vitr-sur-Seine, France) and paclitaxel from Bristol-Myers Squibb (Hounslow, UK). All other drugs, resorufin, ethoxyresorufin, NADP, and isocitric dehydrogenase were purchased from Sigma Chemical Co. (Poole, UK). All chemical reagents were of the highest analytical grade available. Microsomes prepared from control cells and baculovirus-infected insect cells expressing human CYP1B1, CYP1A1, CYP1A2, and CYP2A4 (Supersomes) were purchased from Gentest Co. (Woburn, MA). Human liver microsomes were prepared from surgical resection samples by differential centrifugation.

Ethoxyresorufin O-Deethylase Assay. Ethoxyresorufin O-deethylase activity was measured with cDNA-expressed CYP1B1, CYP1A1, and CYP1A2 (Lubet et al., 1985). Each incubation (2 ml) contained Supersomes (4 pmol of CYP1B1, 1 pmol of CYP1A1, or 1 pmol of CYP1A2), 0.1 M KH₂PO₄, pH 7.4, 200 μl of NADPH-generating system (10 mM NADP, 50 mM isocitric acid, 10 U/ml isocitric dehydrogenase, and 50 mM MgCl₂, pH 7.4), ethoxyresorufin, and inhibitor as appropriate. Supersomes were preincubated before addition of ethoxyresorufin. Reactions were carried out for 10 min (CYP1A1 and CYP1A2) or 20 min (CYP1B1) and terminated by cooling on ice. Fluorescence was measured at excitation λ 550 nm and emission λ 582 nm using a Shimadzu RF-1501 spectrophotometer (Milton Keynes, UK). Control incubations with vector-only microsomes showed no deethylation of ethoxyresorufin. Resorufin production was quantified by reference to a standard curve of authentic metabolite (0–1 μM resorufin in 0.1 M KH₂PO₄, pH 7.4). CYP activity was expressed as picomoles of resorufin formed per minute per picomole of CYP (pmol/min/pmol of CYP).

Inhibition of CYP1B1 by Anticancer Agents. Preliminary incubations were used to screen a variety of anticancer agents for inhibitory potency against CYP1B1 (4 pmol) at 1 μM ethoxyresorufin (Kᵢ = 0.5 μM ± 0.1) and 100 μM putative inhibitor (exceptions: 25 μM ketoconazole, 50 μM paclitaxel, and 50 μM α-naphthoflavone). Apparent Kᵢ values were determined for potent inhibitors at three concentrations of ethoxyresorufin (0.05, 0.1, and 0.5 μM) in the presence of six inhibitor concentrations. The inhibitor concentrations used were as follows: flutamide, mitoxantrone, doxorubicin, daunorubicin, tamoxifen, and vincristine. Each reaction was termi-

Inhibition of EROD by Flutamide. Apparent Kᵢ values were determined for flutamide inhibition of EROD activity (CYP1B1, CYP1A1, and CYP1A2 components). Incubations were carried out at ethoxyresorufin concentrations of 0.05 to 0.5 μM for CYP1B1, 0.05 to 0.2 μM for CYP1A1, and 0.25 to 2.5 μM for CYP1A2, in the presence of flutamide at 0 to 20 μM. Dixon and Cornish-Bowden plots were constructed to model the type of inhibition (Dixon and Webb, 1979).

TABLE 1

<table>
<thead>
<tr>
<th>Inhibition Type</th>
<th>Kᵢ, Mean ± S.D. (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anticancer agents</td>
<td></td>
</tr>
<tr>
<td>Flutamide</td>
<td>Competitive 1.0 ± 0.1</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>Competitive 11.6 ± 0.1</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>Competitive 31.6 ± 9.4</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Competitive 28.0 ± 9.8</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>Noncompetitive 5.0 ± 0.1</td>
</tr>
<tr>
<td>Daunomycin</td>
<td>Mixed 2.6 ± 0.2</td>
</tr>
<tr>
<td>5-Fluorouracil (100 μM)</td>
<td>No inhibition</td>
</tr>
<tr>
<td>Etoposide (100 μM)</td>
<td>No inhibition</td>
</tr>
<tr>
<td>Cyclophosphamide (100 μM)</td>
<td>No inhibition</td>
</tr>
<tr>
<td>Vinblastine (100 μM)</td>
<td>No inhibition</td>
</tr>
<tr>
<td>Vincristine (100 μM)</td>
<td>No inhibition</td>
</tr>
<tr>
<td>Other compounds</td>
<td></td>
</tr>
<tr>
<td>Estradiol</td>
<td>Competitive 1.9 ± 0.1</td>
</tr>
<tr>
<td>Testosterone</td>
<td>Competitive 411.8 ± 40.4</td>
</tr>
<tr>
<td>α-Naphthoflavone</td>
<td>Noncompetitive 0.0025 ± 0.0005</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>Noncompetitive 0.5 ± 0.1</td>
</tr>
<tr>
<td>Cyclosporine (100 μM)</td>
<td>No inhibition</td>
</tr>
<tr>
<td>Erythromycin (100 μM)</td>
<td>No inhibition</td>
</tr>
</tbody>
</table>

Results

Inhibition of CYP1B1. As initial screening for the inhibition of CYP1B1 activity, resorufin production was measured with 1 μM ethoxyresorufin in the presence of 0 μM (as control) or 100 μM anticancer agent. In these conditions, vinblastine, vincristine, 5-fluorouracil, etoposide, and cyclophosphamide did not inhibit CYP1B1 activity (<5%; Table 1). In contrast, flutamide, paclitaxel, mitoxantrone, docetaxel, doxorubicin, daunorubicin, and tamoxifen inhibited CYP1B1 activity by decreasing the production of resorufin by 53 to 99% (data not shown). Further inhibition studies performed with three concentrations of ethoxyresorufin and six concentrations of drugs identified flutamide (Fig. 1), mitoxantrone, docetaxel, and paclitaxel as competitive inhibitors with Kᵢ values of 1.0, 11.6, 28.0, and 31.6 μM, respectively (Table 1). Noncompetitive or mixed inhibition was observed for daunorubicin, doxorubicin, and tamoxifen, and Kᵢ values were 2.1, 2.6, and 5.0 μM, respectively (Table 1).

Similarly, known CYP inhibitors and putative CYP1B1 substrates were also initially screened at 100 μM, and agents with an apparent interaction were further characterized as described above. Erythromycin and cyclosporine did not inhibit CYP1B1 activity (<10%; Table 1). In contrast, testosterone and estradiol were competitive inhibitors of EROD (Kᵢ = 1.9 and 411.8 μM, respectively). Potent noncompetitive inhibition by ketoconazole (Kᵢ = 0.3 μM) and α-naphthoflavone (Kᵢ = 2.8 μM) was observed (Table 1).

Flutamide Metabolism. Flutamide was a potent competitive inhibitor of CYP1B1, suggesting that it is a putative substrate. In vitro incubations of flutamide were performed under the following conditions:
with human liver microsomes or various cDNA-expressed human CYPs. Two flutamide metabolites, FM#1 and FM#2, were produced in the presence of NADP-regenerating system (data not shown). FM#2 was observed after incubation with CYP1B1, CYP1A1, or CYP1A2. FM#2 has been previously identified as 2-hydroxyflutamide following metabolic studies of CYP1A2 (Shet et al., 1997). It is clear from this data that the production of 2-hydroxyflutamide is also under control of CYP1B1 and CYP1A1 activities. As previously reported (Shet et al., 1997), the production of FM#1 is via CYP3A4 activity (Fig. 2).

The 2-hydroxylation of flutamide was produced by cDNA expressing human CYP1B1, CYP1A1, and CYP1A2. Production of 2-hydroxyflutamide by CYP1B1 was described by Michaelis-Menten kinetics, and \( K_m \) and \( V_{max} \) values were calculated by Eadie-Hofstee plots (Fig. 3). Flutamide was a competitive inhibitor of CYP1B1, CYP1A1, and CYP1A2 activities, with \( K_i \) values ranging from 1.0 to 10.3 \( \mu M \) (Table 2). For CYP1B1 and CYP1A2, similar \( K_m \) and \( K_i \) values were obtained (Table 2). In contrast, \( K_m \) and \( K_i \) values for CYP1A1 were 5 and 10 times higher than CYP1B1, respectively (Table 2). \( V_{max} \) values for 2-hydroxylation of flutamide are different for all three enzymes, likely reflecting differences in cytochrome c reductase activity in the microsome preparations (Crespi and Penman, 1997). In the microsome preparation used in this study the cytochrome c reductase activity was 310, 1600, and 2330 nmol/min \( \times \) mg of proteins for CYP1B1, CYP1A1, and CYP1A2, respectively.

**Discussion**

Classical approaches to investigating the mechanisms of metabolism and resistance for anticancer drugs use liver tissue and cancer cell lines, respectively. CYP1B1 is overexpressed in tumor cells, but in the adjacent normal tissues or in human liver no enzyme is detected (Murray et al., 1997). As with most CYPs, CYP1B1 must be induced to be detectable in most human cell lines (Spink et al., 1998). This suggests that CYP1B1 may represent an unrecognized source of metabolism or drug resistance and one which is tumor-selective. We investigated the capability of 12 commonly used anticancer agents to interact with CYP1B1 activity. While 5-fluorouracil, vincristine, vinblastine, etoposide, and cyclophosphamide had no apparent interaction with CYP1B1, seven compounds did merit more extensive evaluation. Flutamide, mitoxantrone, paclitaxel, and docetaxel did show competitive inhibition with \( K_i \) values ranging from 1.0 to 31.6 \( \mu M \) (Table 1), indicating that they are probably substrates of CYP1B1. Flutamide interactions with CYP1B1 were characterized (discussed further below), and mitoxantrone, paclitaxel, and docetaxel biotransformation by CYP1B1 warrants further investigations to clarify its role in regulating the in vivo activity of these agents.

Doxorubicin, daunomycin, and tamoxifen showed mixed or noncompetitive inhibition of CYP1B1, with \( K_i \) values ranging from 2.6 to 5.0 \( \mu M \) (Table 1). This suggests that these agents should not be substrates for CYP1B1 but most likely interact with cofactor binding or other indirect mechanisms. However, the relatively strong affinity of these agents for
CYP1B1 could have a therapeutic significance in patients. For example, agents, such as quinidine and phenobarbital, have enzyme inhibitory or induction characteristics, respectively, on P450 enzymes without being substrates for metabolism.

Testosterone and estradiol showed competitive inhibition of CYP1B1 activity but with differing potency ($K_i = 411.8$ and $1.9 \mu M$, respectively). These results are in line with a previous report, where human CYP1B1-mediated estradiol 4-hydroxylations showed a $K_m$ value of $0.71 \mu M$ (Hayes et al., 1996). Similarly, evidence of testosterone biotransformation mediated by CYP1B1 has been reported (Crespi et al., 1997). This suggests that CYP1B1 activity could play an important role in vivo estradiol metabolism, as it has been hypothesized (Spink et al., 1998). In contrast, with a high $K_v$ value, biotransformation of testosterone, mediated by CYP1B1, is less likely to be of significant in vivo importance.

α-Naphthoflavone appears to be a very potent inhibitor of CYP1B1 with a $K_i$ value ($2.8 \mu M$) 100 times more potent than that identified for ketoconazole (Table 1). The noncompetitive inhibition and the lack of inhibition specificity for α-naphthoflavone and ketoconazole on CYP isoenzymes (Newton et al., 1995) suggest that they probably disturb the interaction between the CYP isoenzymes and its coenzyme(s) or cofactors.

Flutamide metabolism was investigated by in vitro incubations using human liver microsomes or cDNA-expressed human CYPs. Two main flutamide metabolites, FM#1 and FM#2, were produced only in the presence of NADP-regenerating system, consistent with involvement of CYPs. FM#2 has been previously identified as 2-hydroxyflutamide as a product of CYP1A2 activity (Shet et al., 1997). We report here that 2-hydroxyflutamide is also a product of CYP1B1 and CYP1A1 activities (Fig. 2). In contrast, the production of FM#1 is under the control of CYP3A4 activity, but not of CYP1B1, CYP1A1, and CYP1A2 activities, consistent with previous studies (Shet et al., 1997 and Fig. 2).

Kinetic parameters ($K_m$ and $V_{max}$) of flutamide 2-hydroxylation were determined for CYP1B1, CYP1A1, and CYP1A2 (Fig. 3 and Table 2). Additionally, $K_i$ values for flutamide on CYP1B1, CYP1A1, and CYP1A2 were determined (Table 2). For all three CYPs, the inhibition by flutamide is competitive. Differences in the kinetics of flutamide 2-hydroxylation were observed between CYP1B1, CYP1A1, and CYP1A2. Because $K_m$ and $K_i$ values for CYP1B1 and CYP1A2 are similar, 18.6 and 18.0 $\mu M$, and 1.4 and 1.0 $\mu M$, respectively, these results suggest that the metabolic velocity of 2-hydroxyflutamide production, $V_{max}$, should be similar for CYP1B1 and CYP1A2. In contrast, $V_{max}$ for CYP1A2 is 20 times higher than for CYP1B1. However, a recent review highlights that, in contrast to $K_m$ and $K_i$ values, $V_{max}$ determination with cDNA-expressed CYP is highly dependent of the levels of CYP coenzymes, e.g., CYP oxidoreductase (Crespi and Penman, 1997). The amount of CYP isoenzymes is not linearly correlated to the functional (metabolic) activities. This artifact may lead to high variability of $V_{max}$ determination between various cDNA-expressed CYP preparations (Crespi and Penman, 1997).

The CYP1A2 $K_m$ value ($18.6 \mu M$) determined for flutamide hydroxylation in our study is in accordance with previous data using a purified fusion protein containing CYP1A2 ($K_m = 6 \mu M$, Shet et al., 1997). Additionally, Shet and colleagues showed that 2.5 $\mu M$ α-naphthoflavone led to complete inhibition of flutamide biotransformation in human liver microsomes and therefore concluded that CYP1A2 appeared to be the main hepatic metabolizing enzyme involved in flutamide biotransformation. As CYP1B1 is overexpressed in human tumors in contrast to CYP1A2 (McFadyen et al., 1999), our kinetic data strongly suggest that CYP1B1 is the major metabolizing enzyme involved in flutamide 2-hydroxylation in the target cells. This has important potential implications, as 2-hydroxyflutamide appears to have an anti-androgen activity, promoting tumor growth (Shet et al., 1997).

In conclusion, seven structurally diverse anticancer agents have shown inhibitory potency against CYP1B1-mediated EROD activity in the low micromolar range. However, the role of the overexpression of CYP1B1 in tumor in the pharmacological efficiency and drug resistance of these agents in cancer cells needs to be determined. In the past, various mechanisms of drug resistance have been investigated such as efflux systems (e.g., P-glycoprotein) or mutation of the target (Dumontet and Sikic, 1999). It remains to consider that drug metabolism in the cancer cells could also be at the center of the resistance phenotype. The CYP1B1-mediated 2-hydroxylation of flutamide, reported in this article, appears...
to be able to occur in the target cells and may represent a novel mechanism of resistance. Therefore, inhibition of CYP1B1 activity could have significant impact on the clinical outcome of cancer therapy.

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References


Send reprint requests to: Dr. Howard L. McLeod, Washington University School of Medicine, Department of Medicine, Room 1021 CSR/CN, 660 South Euclid Ave., Campus Box 8069, St. Louis, MO 63110-1093. E-mail: hmcleod@imgate.wustl.edu