Selective Biotransformation of Taxol to 6α-Hydroxytaxol by Human Cytochrome P450 2C8

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Abstract

The principal taxol biotransformation reaction of humans and of human hepatic in vitro preparations is 6α-hydroxylation of the taxane ring, but a separate, minor hydroxylation pathway (metabolite B formation) also exists. Taxol metabolism was studied using membrane fractions from Hep G2 cells infected with recombinant vaccinia viruses that contain complementary DNAs encoding several human cytochrome P450 enzymes. Only P450 2C8 formed detectable 6α-hydroxytaxol. Metabolite B formation was catalyzed by complementary DNA-expressed 3A3 and 3A4, but not by 2C8. Each P450 3A preparation catalyzed fedeloxin oxidation. The apparent Kₘ and Vₘₐₓ values for taxol 6α-hydroxylation were 5.4 ± 1.0 μM and 30 ± 1.5 nmol/min/nmol P450, respectively, for complementary DNA-expressed P450 2C8; the values were 4.0 ± 1.0 μM and 0.87 ± 0.06 nmol/min/mg protein, respectively, for human hepatic microsomes. The inhibition of 6α-hydroxytaxol formation by quercetin was competitive with an apparent Kᵢ of 1.3 or 1.1 μM with 2C8 or hepatic microsomes, respectively; retinoic acid was inhibitory, showing an apparent Kᵢ of 27 μM with hepatic microsomes; inhibition by tolbutamide was complex, weak, and unlikely to be clinically relevant. The correlation between hepatic 2C8 protein content and 6α-hydroxytaxol formation was high (r² = 0.82), while the correlation with 2C9 content was low (r² = 0.38).

These data show that human biotransformation routes of taxol result from catalysis by specific enzymes of two P450 families and that taxol 6α-hydroxylation is a useful indicator of P450 2C8 activity in human hepatic microsomes.

Introduction

Taxol is indicated currently for the treatment of refractory ovarian and breast cancer. In contrast to Vinca alkaloids, taxol increases polymerization of microtubules, stabilizes them once polymerized, and inhibits depolymerization. This results in the mitotic arrest of cells in the G₂ or M phases. The major pathway of taxol metabolism in humans and in human-derived in vitro systems is the regiospecific hydroxylation at position six of the taxane ring, with the 6-hydroxyl group trans to the 7-hydroxy group, yielding 6α-hydroxytaxol. This reaction appears to detoxify taxol (1) and, hence, removes pharmacological activity. A second minor pathway for taxol metabolism results in hydroxylation at Ph2 of the C-13 side chain (metabolite B); Refs. 1 and 2; metabolite B formation is catalyzed by the human cytochrome P450 3A subfamily, and the reaction is catalyzed by P450 3A4 (4, 5) but not by P450 3A5 (4). Creestel et al. (5) assigned P450 2C2 enzymes in 6α-hydroxytaxol formation based on a correlation between taxol 6α-hydrolyase activity and total immunodetectable P450 2C (r² = 0.52) determined in hepatic microsomes prepared from 11 individuals. A minimum of four enzymes constitute the human P450 2C subfamily (6), and these enzymes have been shown to have markedly different substrate selectivities (7–9). Because of the complexity of the P450 2C subfamily we have investigated taxol metabolism using cDNA-expressed human cytochrome P450 enzymes and human liver microsomes. Evidence reported herein shows that (a) P450 2C8 efficiently catalyzes taxol 6α-hydroxylation and (b) this enzyme is the principal, if not the only, catalyst of this reaction in humans.

Materials and Methods

Reagents. Taxol (lot FB10110) and baccatin III were obtained from the Developmental Therapeutics Program (National Cancer Institute, Bethesda, MD) and were 99% pure by HPLC. 6α-Hydroxytaxol was isolated from a human bile sample and was pure by HPLC, by extinction coefficient, and by nuclear magnetic resonance (1). Fedoelpine and dehydrofelodipine were gifts from Astra Hässel (Möndal, Sweden). Hydroxylactamutide was a gift from Allan Rettie (Department of Medicinal Chemistry, University of Washington, Seattle, WA). Quercetin was purchased from Sigma Chemical Co. (lot 118P0619). Inhibitory chemicals and the constituents of the NADPH-generating system were purchased from Sigma. NADPH was purchased from Boehringer Mannheim (Indianapolis, IN).

Human Liver Microsomes. Human liver samples, medically unsuitable for liver transplantation, were acquired under the auspices of the Washington Regional Transplant Consortium (Washington, DC), and microsomes were prepared from these samples as described previously (4). Microsomal preparations from human liver 9 were suspended in 0.1 M phosphate buffer containing 1 mM EDTA and 50 μM MgCl₂ (pH 7.4; reaction buffer) to give a final protein concentration of 0.2 mg protein/ml (0.18 nmol total P450/ml). Inhibitors (quercetin, TAO, and retinoic acid) were dissolved in ethanol and added to each microsomal suspension (10 μl/ml) while on ice. Tolbutamide was dissolved in reaction buffer. Reactions (500 μl) were warmed in a shaking water bath for 3 min at 37°C in the presence of an NADPH-generating system (final concentrations: 1 mM NADPH, 1 unit/ml of glucose-6-phosphate dehydrogenase, 10 mM glucose-6-phosphate), 50 μl of 0.2–20 μM taxol in ethanol was added at 0 min, and the reaction was incubated for 15 min.

Microsomes Containing cDNA-expressed P450s. Vaccinia virus expression vectors were constructed and viral stocks were prepared as described previously (10). Hep G2 cells were infected with a recombinant vaccinia virus containing human P450 cDNAs, and the cells were harvested 24 h later. Control Hep G2 cells were infected with wild-type vaccinia virus. The cells were sonicated and centrifuged at 500,000 × g for 12 min. Pellets were suspended in 50 mm potassium phosphate buffer, pH 7.4, and the P450 content was measured by CO difference spectra (11). The amount of expressed P450 used in each incubation reported in Table 1 was (enzyme, pmol): 1A2, 66; 3A3, 100; 3A4, 103; 3A5, 113; 2B6, 95; 2C8, 51; 2C9, 69; and 2C9R144C, 143 human embryoblast.

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The abbreviations used are: 6α-hydroxytaxol, benzenepropanoic acid; β-(benzylamino)-α-hydroxy-6,12b-bis(acetoxy)-12-benzoyloxy) -2a,3,4,4a,5,6,9,10,11,12,12a,12b-dodecahydro-3,4,11-trihydroxy-4a,8,13,13-tetramethyln-1H-cyclodeca(3,4)benz(1,2-b)oxet-9-y1 ester, [2R-[2aR-2aa,3a,4a,8a,8b,9a(α*,β*)], 11a,12a,12b,12aa] 6α-hydroxytaxol; metabolite B, benzenepropanoic acid; β-(benzylamino)-α,4-dihydroxy-6,12b-bis(acetoxy)-12-benzoyloxy)-2a,3,4,4a,5,6,9,10,11,12,12a,12b-dodecahydro-4,11-dihydroxy-4a,8,13,13-tetramethyln-1H-cyclodeca(3,4)benz(1,2-b)oxet-9-y1 ester, [2αR-[2aa,4a,8a,8b,9a(α*,β*)], 11a,12a,12a,12aa] cDNA, complementary DNA; HPLC, high performance liquid chromatography; SIMS, secondary-ion mass spectrometry; TAO, troleandomycin; TC, TK + 143 human embryoblast.

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TAXOL BIOTRANSFORMATION BY P450 2C8

Table 1 Formation of taxol metabolites by cDNA-expressed human P450 enzymes

<table>
<thead>
<tr>
<th>cDNA-expressed enzyme*</th>
<th>Catalytic activity (nmol/min/nmol P450)</th>
<th>Metabolite B</th>
</tr>
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<tbody>
<tr>
<td>Control cells</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1A2</td>
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<td>3A3</td>
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<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2C8</td>
<td>10.4</td>
<td>ND</td>
</tr>
<tr>
<td>2C9</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2C9R144C</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Amount of P450 ranged from 51 to 113 pmol/incubation. Control activities for P450 3A enzymes were determined with felodipine (P450 form, nmol product/min/nmol P450): 3A3, 3.5; 3A4, 3.5; 3A5, 2.7.

100. Subsequent experiments with 2C8 used 10 pmol P450/incubation. Taxol (2–200 μM) and inhibitors were added to microsomal suspensions (4.5 ml reaction), and the reaction mixtures were warmed for 3 min at 37°C in a shaking water bath. Reactions were initiated by adding 500 μl of 1 mM NADPH and were terminated 30 min later.

Sample Preparation, Chromatography, and Metabolite Structural Analysis. All reactions were terminated by placing the tubes on ice and by adding 5.0 ml of 2.0 μM baccatin III (internal standard) dissolved in acetonitrile to each tube. Tubes were vortexed and centrifuged, and the supernatant was evaporated to dryness with a Speed-Vac apparatus. All samples were dissolved in 200 μl of 1:1 acetonitrile:water to each tube. The residue from solvent evaporation was dissolved in 200 μl of 1:1 acetonitrile:water (v/v), 5 ml chloroform was added, the organic layer was washed twice with 10 ml of 0.02 N NaOH and twice with 10 ml of water, and the chloroform was evaporated with a stream of air; control reactions for these experiments were prepared similarly. The residue that resulted from solvent evaporation was dissolved in 200 μl of 1:1 acetonitrile:water, and 2 ml of acetonitrile was added; tubes were vortexed and centrifuged, and the supernatant was evaporated to dryness with a Speed-Vac apparatus.

Assessment of Reaction Kinetics. Inhibition kinetics were analyzed using the methods and programs of Cleland (12). Km and Vmax values for human liver microsomes and expressed enzymes were determined by least-squares fit to a hyperbola. Inhibition constants were determined from the best fit to competitive, noncompetitive, and uncompetitive equations.

Protein Electrophoresis and Western Immunoblotting. Protein electrophoresis and Western immunoblotting were conducted following standard protocols and used rabbit anti-P450 IgG produced against rat P450 2C7 (13). This antibody is known to cross-react with human P450 2C8 and human P450 2C9 (13). For Western blot standards, TK- cells were infected with cDNAs encoding P450 2C8 and P450 2C9 for 48 h. Total cell lysate protein (containing 5 pmol P450 2C8 or 4 pmol P450 2C9) from TK- cells or microsomal protein prepared from nine human livers (63 μg) were loaded in each lane. Western blots were analyzed and integrated by densitometric scanning using a Molecular Dynamics computing densitometer and ImageQuant software. Integrated areas were determined after manually setting the baseline. Variation in the extent of antigen staining on separate western blots was corrected for by comparing the integrated areas of identical samples analyzed on each blot. Comigration of 2C8 from TK- cells and the second fastest migrating antigen band of human microsomes was established by mixing these samples.

Results

Analysis of the P450 2C8 Taxane Product. HPLC and SIMS analyses indicate that the product of taxol incubation with expressed human P450 2C8 is 6α-hydroxytaxol. The product was inseparable from authentic 6α-hydroxytaxol when studied with three HPLC protocols, and the relationship between the peak integral and the amount of taxane added was linear in each case (data not shown). Positive-ion SIMS showed molecular ions of m/z 854 or of 870 for taxol or the 2C8 product, respectively. Negative-ion SIMS showed ions at m/z 852 and 525 for taxol and ions at m/z 868 and 541 for the 2C8 product; these ions represent molecular ions and taxane-ring fragments of these chemicals (1, 2, 14). B/E linked-scan mass spectra were obtained for taxol (positive ions: m/z 794, 569, 509, 286, and 268; negative ions: m/z 792, 525, and 284) and for the 2C8 product (positive ion: m/z 810, 585, 525, 286, and 268; negative ion: m/z 808, 541, and 284); assignments for each of these fragments have been given for taxol (14).

Form Selectivity and Reaction Kinetics. cDNA-expressed human cytochrome P450 2C8 catalyzed the biotransformation of taxol to 6α-hydroxytaxol (Table 1); neither P450 2C9, its variant P450 2C9R144C, nor any other expressed P450 enzyme studied produced detectable 6α-hydroxytaxol (Table 1). Tolbutamide methylhydroxylation was used as a positive control for each P450 2C preparation, and these enzymes did catalyze the reaction (data not shown). The formation of metabolite B (2, 4) was catalyzed by P450 3A3 and P450 3A4, but not by P450 3A5 (Table 1). Felodipine oxidation was used as a positive control for each P450 3A preparation, and these enzymes did catalyze the reaction (data not shown). The formation of metabolite B (2, 4) was catalyzed by P450 3A3 and P450 3A4, but not by P450 3A5 (Table 1). Felodipine oxidation was used as a positive control for each P450 3A preparation, and these enzymes did catalyze the reaction (data not shown).

Characteristics of Inhibition. Quercetin was shown previously to be a strong inhibitor of taxol 6α-hydroxylase activity (3, 4), and was shown not to affect P450 3A4-mediated metabolite B formation (4). The data in Table 2 shows that quercetin is a competitive inhibitor of 6α-hydroxytaxol formation catalyzed by cDNA-expressed 2C8 and human hepatic microsomes, with Ki values of 1.3 and 1.1 μM, respectively. Retinoic acid, another P450 2C8 substrate (15), inhibited taxol 6α-hydroxylation with an apparent Ki value of 27 μM when studied with human liver microsomes (Table 2). The inhibition of 6α-hydroxytaxol formation by tolbutamide was weak and showed...
both competitive and noncompetitive components (Table 2). In addition, 35 μM taxol inhibited tolbutamide metabolism by 75% when studied with expressed P450 2C8 (300 μM tolbutamide).

Because TAO was a strong inhibitor of human taxol biotransformation but was selective for P450 2C8-mediated metabolite B formation (4), the effect of TAO on the 2C8-mediated reaction was tested. TAO did not inhibit taxol 6α-hydroxylation when added at 2, 50, or 200 μM and when the taxol concentration was 10 μM; this finding with expressed 2C8 is consonant with the inhibitory effect of TAO on taxol metabolism by human hepatic microsomes (4, 5).

Correlation Analyses. Western immunoblot analysis was performed using a rabbit antibody preparation raised against rat P450 2C7, and this preparation recognizes human P450 2C8 and P450 2C9 proteins (13). Bands at M, 51,000 and 55,000 were found in each of the nine liver microsomal preparations (Fig. 1), and these bands were assigned as P450 2C8 and P450 2C9, respectively, based on these observations: (a) similar molecular weights for P450 2C8 and P450 2C9 purified from human liver have been reported (16); (b) expressed 2C8 or 2C9 gave bands that comigrated with the hepatic proteins (Fig. 1); and (c) no band separation occurred when 2C8 was mixed with human hepatic microsomes. The relative staining of 2C8 or 2C9 bands among liver samples from 9 individuals (4) was assessed by densitometry. Fig. 1 shows that the correlation between P450 2C8 content and in vitro synthesis of 6α-hydroxytaxol was high (r² = 0.82), whereas the relationship between P450 2C9 content and 6α-hydroxytaxol formation was poor (r² = 0.38).

Discussion

The results of the experiments described here indicate that P450 2C8 is the predominant enzyme of those examined responsible for formation of the principal human metabolite and detoxication product of taxol (1), 6α-hydroxytaxol. This conclusion and a supporting data set (4) are in variance with another report on human 6α-hydroxytaxol formation (3). The conclusion of 2C8-selective catalysis is derived from the lines of evidence that follow: (a) of several cDNA-expressed P450 enzymes tested, only P450 2C8 showed a capacity for taxol 6α-hydroxylation; (b) cDNA-expressed 2C8 was an efficient catalyst of the reaction, as demonstrated by a turnover number of 30 nmol/min/nmol P450; (c) taxol 6α-hydroxylation activity correlated well (r² = 0.82) with hepatic P450 2C8 content, but did not correlate (r² = 0.38) with the hepatic content of P450 2C9; (d) the apparent Kₘ value obtained for taxol 6α-hydroxylation by expressed P450 2C8 (5.4 ± 1.0 μM) was similar to that found with a human liver microsomal preparation (4.0 ± 1.0 μM); and (e) the inhibition kinetics for quercetin were virtually identical for expressed P450 2C8 and for hepatic microsomes with apparent Kᵢ values of 1.3 and 1.1 μM, respectively.

Metabolite B formation was catalyzed by P450 3A3 and by P450 3A4 with comparable rates, but P450 3A5 appeared incapable of catalyzing taxol biotransformation (Table 1). These data are another example of frank substrate selectivity differences between P450 3A4 and the polymorphic P450 3A5 (6).

Quercetin [and other flavonoids (kaempferol, naringenin]; Ref. 4] is shown here to inhibit a P450 2C8 marker activity; hence, flavonoids can be inhibitory toward P450 1A (17), P450 3A (18), and P450 2C subfamily catalysis. Mephenytoin, tolbutamide, hexobarbital, and sulfaphenazole did not affect the formation of 6α-hydroxytaxol by human hepatic microsomes (4), however, although they are common inhibitors of enzymes in the P450 2C subfamily. These inhibition data...
are consistent with the extant literature and support the assignment of 2C8 in taxol 6α-hydroxylation because: (a) mephenytoin is a selective substrate for P450 2C19, and 2C8 apparently does not catalyze mephenytoin biotransformation (7); (b) the $K_m$ for tolbutamide hydroxylation by expressed 2C8 preparations is 450–650 μM (9, 19), and there is general agreement that P450 2C9 predominates in this reaction (7, 9); (c) tolbutamide is inhibitory toward bona fide 2C9-catalyzed reactions (19, 20), in contrast to tolbutamide effects on taxol 6α-hydroxylation; (d) hexobarbitual is a substrate for 2C9, 2C10, and possibly for 2C19 (6); and (e) sulfaphenazole selectively inhibits 2C9 (9).

There exists only a limited knowledge of 2C8 substrates or inhibitors given to humans (6), but some inhibitors of taxol 6α-hydroxylation are potential chemotherapeutic agents. Quercetin inhibits the growth of several human tumor-derived cell lines (including leukemia, Ehrlich’s ascites tumors, squamous cell carcinoma of head and neck origin, gastric and colon carcinoma, the estrogen receptor-positive breast carcinoma MCF-7; Ref. 21), and quercetin is being assessed in Phase I clinical trials. All-trans retinoic acid is a promising experimental agent for acute promyelocytic leukemia. Retinoic acid and retinol are substrates of 2C8 (15), and retinoic acid does inhibit taxol 6α-hydroxylation (this work). If retinoic acid or quercetin are coadministered with taxol, then these data could be useful in assessing the potential for a pharmacokinetic interaction. Moreover, because of its higher affinity for 2C8 compared to other known substrates (9, 15, 19) and because of the relatively high turnover number of 6α-hydroxylation, taxol may serve as a useful in vitro probe of human P450 2C8 activity.

Acknowledgments

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References


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