Thalidomide Metabolism by the CYP2C Subfamily

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ABSTRACT

Purpose: This research investigated the biotransformation of thalidomide by cytochrome P-450 (CYP).

Experimental Design: We used liver microsomes from humans and/or animals and the recombinant specific CYP isoforms to investigate CYP-mediated metabolism of thalidomide.

Results: Thalidomide was biotransformed into 5-hydroxythalidomide (5-OH) and diastereomeric 5'-hydroxylthalidomide (5'-OH) by liver microsomes. The human liver microsomes with higher CYP2C19 activity formed more metabolites than those with lower CYP2C19 activity and had less activity in metabolite formations than those from rats. Recombinant human CYP2C19 and rat CYP2C6 isoforms were primarily responsible for forming these metabolites, and the male rat-specific CYP2C11 formed only 5'-OH. 5-OH was subsequently hydroxylated to 5,6-dihydroxythalidomide by CYP2C19, CYP2C9, and CYP1A1 in humans and by CYP2C11, CYP1A1, CYP2C6, and CYP2C12 in rats. Incubations with S-mephenytoin and omeprazole, substrates of CYP2C19, inhibited metabolism by human liver microsomes, supporting the involvement of CYP2C19. α-Naphthoflavone, an inhibitor of CYP1A1, simultaneously stimulated the 5-OH formation and inhibited cis-5'-OH formation catalyzed by human liver microsomes. The contribution of the CYP2C subfamily was supported by the immunoinhibition study using human liver microsomes. When we used the microsomes from treated rats, the metabolite formations did not increase by inducers for CYP1A1, CYP2B, CYP2E, CYP3A, or CYP4A, suggesting that these could not be involved in the main metabolic pathway in rats.

Conclusions: We discovered that the polymorphic enzyme CYP2C19 is responsible for 5- and 5'-hydroxylation of thalidomide in humans. In rats, thalidomide was hydroxylated extensively by CYP2C6 as well as the specific enzyme CYP2C11.

INTRODUCTION

Thalidomide is an old compound that was originally developed as a sedative and was eventually pulled from the general market because of its catastrophic adverse effect of teratogenesis. It has been reintroduced over the past several years in the treatment of leprosy and, more recently, has been found to be effective against various diseases including multiple myeloma and prostate cancer, at least in part, through the inhibition of angiogenesis (1–3).

There have been numerous investigations of thalidomide, including the molecular target of the pharmacological action (1, 4–8), and it has been known that thalidomide requires microsomal CYP-catalyzed biotransformation for its pharmacological activity in terms of toxicity to lymphocytes, inhibition of cellular adhesion, alternation of cell morphology and differentiation, and antiangiogenesis (4, 9–13). None of the breakdown products generated from the nonenzymatic hydrolysis process are responsible for the activity (14). Because human fetal tissues have significant drug-metabolizing enzymatic activity, even at early stages of gestation (15, 16), such intracellular metabolism could produce metabolites or reactive intermediates from thalidomide, causing teratogenic events. Thalidomide has been shown to generate reactive oxygen species through prostaglandin H synthase, which may be responsible for the teratogenicity of thalidomide using the rabbit model (5, 7). An interspecies difference in sensitivity to thalidomide has also been explained by the higher embryonic DNA oxidation from the reactive oxygen species in the rabbit model than in the mouse model that is believed to be resistant to the thalidomide teratogenicity (7, 17, 18). Because the newborn rabbits as well as other rodents, unlike human fetuses, essentially lack CYP enzyme activities, other enzyme systems, including prostaglandin H synthase, may play an important role in xenobiotic metabolism in the animal fetus (19, 20). Therefore, even if the prostaglandin H synthase system would be deeply related to the teratogenicity of thalidomide in the animal fetus, the findings do not preclude a role of the CYP-mediated metabolism in the human fetus and adult animal. As a result, we cannot simply extrapolate the ways in which the mechanisms occur in humans and adult animals from the experiments using fetal animals. Despite numerous attempts made over a period of >40 years to elucidate how thalidomide could act in humans, the true mechanisms still remain ambiguous and controversial.

The main transformation of thalidomide in humans is spontaneous nonenzymatic hydrolysis (21). Indeed, thalidomide undergoes very little metabolism by the CYP system in vitro, and as far as CYP3A4, there was little in vivo drug interaction between thalidomide and hormonal contraceptives (22–24). However, at least two hydroxylated metabolites have been
found in human urine or plasma, which could be formed at very low concentrations after incubation with human liver microsomes or 9000 × g supernatant fractions in vitro (22, 23). The two metabolites are 5-OH, formed by hydroxylation of the phthalimide ring possibly via arene oxides, and 5’-OH, by hydroxylation of the glutarimide ring leading to diastereomeric products because a new chiral center is formed (Fig. 1). It has also been suggested that at least two distinct metabolites or intermediates of thalidomide via a CYP system should exist with and without involvement of arene oxide formation, but at present there is no evidence that these are relevant to 5-OH and 5’-OH (25).

The fundamental questions that remain unanswered are: does thalidomide undergo CYP metabolism, and if so, what isozymes are involved? Identifying the specific isozyme is critical to understanding the pharmacokinetic/pharmacodynamic variation in thalidomide-treated patients, especially when activity and/or expression of the enzyme is polymorphic. It is also useful to predict drug interactions, which may occur with simultaneously administered drugs. Pharmacogenetics analysis, the study of genetically determined alternations in a metabolic pathway of a drug, could stratify a subgroup of patients with different pharmacokinetic/pharmacodynamic profiles and underlying genetic markers (e.g., single nucleotide polymorphisms) optimizing drug doses or schedules based on each patient’s genotype. A few investigators have attempted to find pharmacogenetic risk factors of thalidomide neuropathy in small genotype. A few investigators have attempted to find pharmacogenetic risk factors of thalidomide neuropathy in small populations (26, 27). Thus, identifying the pathway should provide us with a useful guide in the pharmacogenetic investigation of thalidomide.

This research was undertaken to elucidate biotransformation of thalidomide by human and/or animal CYP and the specific CYP isozyme, if any. Because there is in vitro evidence that thalidomide requires metabolic activation by CYP and the metabolites of thalidomide can be identified in urine or plasma, understanding the metabolic pathway could indicate the ways in which thalidomide works, aiding us in our search for a more effective and reliable therapy and a development of more effective new agents.

**MATERIALS AND METHODS**

**Chemicals and Reagents.** Racemic thalidomide (100%) was provided by Celgene Corp. (Warren, NJ). Hydroxylated compounds of thalidomide (cis-5’-OH, 4,5-dihydroxythalidomide, 5,6-dOH, and N-hydroxythalidomide) were provided by Dr. K. Eger (University of Leipzig, Leipzig, Germany) and Dr. P. K. Li (Ohio State University, Columbus, OH; 5-OH and 4-hydroxythalidomide). S-(+)-Mephenytoin and antihuman CYP antibodies were purchased from Gentest Corp. (Woburn, MA). Other chemicals were purchased through Sigma-Aldrich Co. (St. Louis, MO).

**Human and Animal Liver Microsomes and Microsomal Fraction Specifically Expressing CYP.** Pooled human and individual liver microsomes were purchased from XenoTech, LLC (Kansas City, KS; n = 16) and Gentest Corp. (n = 10, individual donors H112 and H42). The microsomes from H112 and H42 have higher and lower S-mephenytoin 4’-hydroxylation activity, a prototype reaction catalyzed by CYP2C19: 262, 8, and 66 pmol/min/mg for H112, H42, and the pooled microsomes, respectively. The genotype of CYP2C19 from donors H42 and H112 was homozygous for the reference sequences.

Pooled microsomes prepared from untreated male animals and those from male Sprague Dawley rats treated with prototypical CYP inducers were provided by XenoTech, LLC. The male animals included Sprague Dawley rats (n = 200), Fischer 344 rats (n = 20), CD1 mice (n = 400), New Zealand rabbits (n = 6), and Beagle dogs (n = 6). The treated rats received isoniazid (200 mg/kg; n = 21), dexamethasone (50 mg/kg; n = 23), clofibric acid (200 mg/kg; n = 16), or saline (5 ml/kg; n = 42) once/day over a period of 4 days or a single injection of Aroclor 1254 (500 mg/kg; n = 15) on day 1, followed by preparations of the liver microsomes on day 5. The anticipated induction of each CYP activity was confirmed by the vendor: Aroclor 1254, >10-fold of CYP1A1 and CYP2B; isoniazid, 2–3-fold of CYP2E1; dexamethasone, 3–5-fold of CYP3A; and clofibric acid, >10-fold of CYP4A. Another batch of pooled microsomes from male and female Sprague Dawley rats (n = 15) were purchased from Gentest Corp.

Recombinant CYP enzymes containing baculovirus-expressed CYP were purchased from Gentest Corp. The microsomal fractions expressing CYP1A1, -1A2, -1B1, -2A1, -2D6*1 (-2D6), or -3A5 for human enzymes and CYP1A1, -1A2, -2D1, or -2D2 for rats were coexpressed with NADPH-CYP oxidoreductase. Those expressing CYP2A6, -2B6, -2C8, -2C9+1-Arg144 (-2C9), -2C19, -2E1, -3A4, or -3A7 for humans and CYP2A2, -2B1, -2C6, -2C11, -2C12, -2C13, -3A1, or -3A2 for rats were coexpressed with cytochrome b5 together with NADPH-CYP oxidoreductase. Control microsomes were prepared from insect cells infected with wild-type baculovirus.

**Incubation with Microsomes and HPLC Analyses of Thalidomide Metabolites.** A typical reaction mixture consisted of 100 mM Tris-HCl buffer (pH 7.5), a NADPH-generating system (0.2 mM NADP+, 8 mM MgCl2, 4 mM glucose-6-phosphate, and 1.2 units/ml glucose-6-phosphate dehydrogenase), a desired concentration of substrate, and mi-
crosomal protein in a final volume of 200 μl. Thalidomide and
the metabolite were dissolved in DMSO (final concentration
of DMSO was <1%), and the concentrations at the beginning of
incubations were usually 400 μM for thalidomide and 20 μM for
the metabolites. In the preliminary experiments, the substrate
concentration of 400 μM for thalidomide and of 20 μM for 5-OH
was decreased by <10% after a 50-min incubation at 37°C in a
shaking water bath. The microsomal amount was usually 0.8
mg/ml for liver microsomes and 100 pmol CYP/ml for the
recombinant enzymes. Polypropylene tubes were used for the
incubation with liver microsomes or by adding an enzyme.
Supernatant (320 μl) was injected into a Waters Nova-Pak C18 HPLC column (3.9 × 300-mm, 4 μm), separated
at room temperature at a flow rate of 1 ml/min, and detected at
UV wavelengths of 220 nm for cis-5’-OH, 236 nm for 5-OH,
and 248 nm for the internal standard and 5,6-OH (Hewlett
Packard 1100 Series). A mobile phase consisted of 0.1 M
NaH2PO4 buffer (pH 3.0) and acetonitrile (Table 1): method A
was used for quantitative analysis of 5-OH; method B for cis-5’-OH;
and method C for 5,6-dOH and other possible metabolites.
Average retention times were 5.1 min for 5-OH in method A,
6.0 min for cis-5’-OH in method B, and 15.1 min for 5,6-dOH
in method C. The limits of quantification were 8 nM for 5-OH,
cis-5’-OH, and 5,6-dOH. The intra- and interassay coefficients
of variation at 10 nM were 1.2% (n = 3) and 12.0% (n = 9)
for 5-OH, 2.2% (n = 3) and 10.5% (n = 8) for cis-5’-OH, and 1.0%
(n = 3) and 3.5% (n = 4) for 5,6-dOH, respectively.

Exact kinetic parameters for the metabolite formations
were not calculated because of hydrolysis of the generated
metabolites, epimerization between cis-5’-OH and trans-5’-OH,
and the subsequent 6-hydroxylation of 5-OH. In preliminary
experiments, 5-OH, cis-5’-OH, and 5,6-dOH at concentrations
of 0.01 μM were decreased by 3, 32, and 19%, respectively, after
50-min incubations of reaction mixture without a NADPH-
generating system. However, because the rate of cis-5’-OH
formation was linear, only tentative K values were obtained using
Lineweaver-Burk plots measured at thalidomide concentrations
between 50 and 600 μM.

All incubations and determinations were performed sepa-
rately using each metabolite. Results are presented as means
with SD from three independent experiments performed in
duplicate or as representative means of duplicate. All paired or
unpaired t tests were two-tailed.

**Inhibition Studies.** Thalidomide was incubated alone or
with a CYP-specific chemical inhibitor to estimate the compet-
itive inhibitory effect on 5-OH and cis-5’-OH formations (29, 30).
The chemicals were dissolved in methanol (orphenadrine,
quercetin, sulfaphenazole, 5-mephenytoin, omeprazole, and
toconazole) or in DMSO (α-naphthoflavone). The final concen-
trations of all organic solvents in the incubation mixture were
uniformly 0.2%. The metabolite formations with the inhibitors
were compared with the controls of methanol or DMSO as the
vehicle. The effect of omeprazole on cis-5’-OH formation by
human liver microsomes could not be evaluated because the
possible metabolite peak from omeprazole in human liver mi-
crosomes, as well as recombinant CYP3A4 enzyme, overlaid
with that of cis-5’-OH. The concentrations of the inhibitor were
determined to cover the Ki for each prototype reaction in
human liver microsomes (29).

The immunoinhibition of 5-OH and cis-5’-OH formations
was examined by preincubation of the human liver microsomes
from donor H112 with preimmune IgG or anti-CYP IgG (0.5
and 4.0 mg IgG/nmol CYP) under the conditions recommended
by the vendor, using antisera for CYP2C13 (polyclonal),
CYP1A1/1A2 (polyclonal), or CYP2D6 (monoclonal). Accord-
ing to the vendor’s assay, the antiserum for CYP2C13 nonse-
lectively inhibits all human CYP2C isozymes.

**RESULTS**

**Metabolite Formations from Thalidomide and 5-OH by**
**Human and Rat Liver Microsomes.** 5-OH, diastereomeric
cis- and trans-5’-OH, and unknown metabolite(s) were found
after incubating thalidomide (400 μM) for 50 min with 0.8
mg/ml human or rat liver microsomes in the presence of a
NADPH-generating system (Table 2; Fig. 2). The final concen-
tration of 5-OH was <0.02 μM in the human liver microsomes
from donor H112. The peaks of 5-OH and cis-5’-OH were
corroborated by the same migration in the chromatograms and
the equivalent UV spectrums as the authentic standards. The
metabolite trans-5’-OH had the same migration as an epimer-
ized product converted from cis-5’-OH after hydrolysis (31).
The retention time (14.2 min in method C) of the unknown
metabolite(s) differed from that of N-hydroxythalidomide (18.8
min in method C), 4-hydroxythalidomide (16.0 min in method
C), 4,5-di-hydroxythalidomide (14.6 min in method C), or
potential metabolites from incubations of these compounds as
substrates. A structural elucidation of the unknown metabo-
lite(s) was not attempted.

The formations of 5-OH and cis-5’-OH from thalidomide
by human liver microsomes were less than one-tenth of those by
rat microsomes (Table 2). Microsomes from donor H112, who

<table>
<thead>
<tr>
<th>Method</th>
<th>Acetonitrile (v/v, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>25 (0–8 min), 80 (8–9 min), 25 (9–13 min)</td>
</tr>
<tr>
<td>B</td>
<td>20–25 (0–10 min), 25 (10–11 min), 80 (11–12 min), 20 (12–16 min)</td>
</tr>
<tr>
<td>C</td>
<td>6 (0–5 min), 6 to 25 (5–11 min), 25 (11–19 min), 80 (19–20 min), 6 (20–23 min)</td>
</tr>
</tbody>
</table>
has higher CYP2C19 activity, formed measurable amounts of 5-OH, but those from the pooled and donor H42 did not. The 5-OH formation by female rats was higher than that found in the male rats ($P < 0.01$, paired $t$ test; $n = 3$), and the cis-5'-OH formation by female rats was lower than male rats ($P = 0.02$, $t$ test; $n = 3$). The rates of cis-5'-OH formation from thalidomide increased linearly with microsomes up to 1.0 mg/ml liver microsomes from rats (data not shown) and with time up to 50 min; those of 5-OH formation were not linear (Fig. 3). The tentative $K_m$'s of 5'-OH formation were 380 ± 40 ($n = 3$) and 310 ± 10 µM ($n = 3$) for male and female rats, respectively (Fig. 4).

Formation of 5,6-dOH from 5-OH was subsequently confirmed after a 50-min incubation of 5-OH (initially 20 µM) as a substrate with 0.8 mg/ml human or rat liver microsomes (Table 2, Fig. 2). Similar to the metabolites from thalidomide, the 5,6-dOH formation by human liver microsomes was less than one-tenth of that of the male rats. The 5,6-dOH formation by male rats was >5 times higher than by female rats ($P < 0.01$, paired $t$ test; $n = 3$). A small peak with the same retention time as 4,5-dihydroxythalidomide was formed from 5-OH by the male rat liver microsomes but was not quantifiable. No apparent metabolites were formed from cis-5'-OH after incubations with human or rat liver microsomes.

**Metabolite Formations by Induced Rats and Interspecies Comparison.** The metabolite formations were compared using the microsomes from rats treated with the CYP-specific inducer to assess the isozyme responsible for metabolism (Table 3). The treatment with each CYP inducer did not increase the formations of 5-OH, cis-5'-OH, and 5,6-dOH as much as marker activities of the induction indicated. These three metabolite formations by the microsomes from isoniazid- and dexamethasone-induced rats and the 5,6-dOH formation by Aroclor 1254-treated rats were less than those found in the controls.

Among the animals evaluated, the microsomes from humans formed the least amounts of 5-OH and cis-5'-OH. The 5-OH formation by CD1 mice microsomes was >20 times after a 50-min incubation compared with that by humans (Table 4; Fig. 3).

**Table 2** Metabolite formations by human and rat microsomes

<table>
<thead>
<tr>
<th>Microsomes</th>
<th>Total CYP* (nmol/mg)</th>
<th>5-OH</th>
<th>cis-5'-OH</th>
<th>5,6-dOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pooled</td>
<td>420</td>
<td>&lt;0.20</td>
<td>0.61 ± 0.10</td>
<td>0.55 ± 0.07</td>
</tr>
<tr>
<td>H112</td>
<td>651</td>
<td>0.36 ± 0.06</td>
<td>0.87 ± 0.26</td>
<td>0.56 ± 0.09</td>
</tr>
<tr>
<td>H42</td>
<td>371</td>
<td>&lt;0.20</td>
<td>0.50 ± 0.20</td>
<td>0.41 ± 0.05</td>
</tr>
<tr>
<td>Rats</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pooled male</td>
<td>550</td>
<td>2.09 ± 0.57</td>
<td>9.23 ± 1.33</td>
<td>11.70 ± 1.44</td>
</tr>
<tr>
<td>Pooled female</td>
<td>670</td>
<td>2.39 ± 0.54</td>
<td>5.45 ± 0.81</td>
<td>1.92 ± 0.31</td>
</tr>
</tbody>
</table>

* The CYP contents and CYP2C19 activity (S-mephenytoin 4'-hydroxylation) were provided from the vendor. The CYP2C19 activity was 66, 262, and 8 (pmol/min/mg) for the microsomes from pooled human, individual donors H112 and H4, respectively.

* Results are presented as mean with SD from independent three experiments performed in duplicate.

**Fig. 2** HPLC chromatograms of metabolites after 50-min incubations of thalidomide (400 µM) or 5-OH (20 µM) with human liver microsomes (0.8 mg/ml, H112). Amounts of the metabolites are close to the quantifiable limits. A, metabolites from thalidomide in the chromatogram of method C. An unknown metabolite was seen at 14.2 min just before the hydrolysis product of thalidomide. Peaks of trans-5'-OH and 5-OH were overlapped. B, metabolites from thalidomide in method A. C, metabolites from thalidomide in method B. D, metabolites from 5-OH in method C. I.S., internal standard; mAU, milli arbitrary unit(s).

**Metabolite Formations by Recombinant CYP Enzymes.** When thalidomide was incubated with the human CYP isoforms, CYP2C19 formed both 5-OH and cis-5'-OH (Table 5). A lesser amount of cis-5'-OH was formed by CYP2B6. The cis-5'-OH formation by CYP2C9 was detected, although this was below the quantification limit. Among the rat CYP isoforms, CYP2C6 showed high activity of both metabolite formations. CYP2C11, the male-specific isozyme (32, 33), formed a large amount of cis-5'-OH but did not form the measurable 5-OH. Moderate activities for both metabolite formations by CYP2D2 were found. When 5-OH was incubated as a substrate, CYP2C19, followed by CYP1A1 and CYP2C9 in human isozymes, had activity for 5,6-dOH formation. Among the rat isoforms, CYP2C11 had the highest activity, followed by CYP1A1, CYP2C6, and CYP2C12. The isozymes involved in the 5,6-dOH formation were less specific, and the formations by CYP1A2 (human and rat), -2D2, -2D6, -2C18, -3A1, -3A2, and -3A4 were detectable but under the quantification limit. All of the remaining isozymes showed similarly low activities of the metabolite formations as those of the control microsomes.

The increase of 5-OH formation with 100 pmol/ml micro-
somes of CYP2C19 was linear with time until 50 min (Fig. 3). The 5-OH formation by CYP2C6 increased with time; however, it did not demonstrate clear linearity. The cis-5'OH formation from thalidomide was increased linearly with microsomes up to 125 pmol/ml and with time up to 50 min with 100 pmol/ml microsomes of CYP2C19, CYP2C6, or CYP2C11. The tentative K_m so f cis-5'OH formations were 300 (n = 3) and 31070 M (n = 3) for CYP2C6 and CYP2C11, respectively (Fig. 4).

**Inhibition Studies.** Incubation with S-mephenytoin, a prototype substrate of CYP2C19 in humans (29, 34, 35), inhibited 5-OH and cis-5'OH formations by the human and rat liver microsomes, which were consistent with the results of the inhibition studies using recombinant enzymes CYP2C19, CYP2C6, and CYP2C11 (Table 6). Omeprazole, potent inhibitor for CYP2C19 (29), also strongly inhibited 5-OH formation of human liver microsomes, <5% of the control by omeprazole of 2.5 μM. Orphenadrine, a relatively specific inhibitor of CYP2B6, also inhibited the metabolite formations, especially by the rat liver microsomes (30). The inhibitory effects of orphenadrine on the formations of 5-OH and cis-5'-OH by CYP2C19, CYP2C6, and CYP2C11 were validated using the recombinant enzymes. The inhibitory effects of sulfaphenazole and quercetin, inhibitors of CYP2C9 and CYP2C8, respectively, were little or weak.

In contrast, α-naphthoflavone stimulated 5-OH formation but not cis-5'-OH formation by human liver microsomes (Table 6). When we used the H112 microsomes, 10 μM α-naphthoflavone increased the rate of 5-OH formation from 0.24 ± 0.02 (DMSO as a vehicle) to 0.67 ± 0.06 pmol/min/mg (P < 0.01, paired t test; n = 3). The pooled human liver microsomes (Gentest Corp.) formed quantifiable amounts of 5-OH with 10 μM α-naphthoflavone (0.22 ± 0.03 pmol/min/mg; n = 3) as did the recombinant CYP3A4 enzyme (with NADPH-CYP oxidoreductase and cytochrome b_5, 4.1 pmol/min/nmol CYP). There was no metabolite from α-naphthoflavone that had a similar retention time as 5-OH on the HPLC chromatogram after incubating the chemical alone with human liver microsomes or the CYP3A4 enzyme. These increased 5-OH formations were inhibited to the levels below quantification by the addition of 1 μM ketoconazole, an inhibitor for CYP3A4. The rat microsomes and the recombinant CYP2C19 and CYP2C6 enzymes were moderately inhibited from 5-OH formations by α-naphthoflavone.

In the presence of the anti-CYP2C antibodies of 4.0 mg IgG/nmol CYP, both 5-OH and cis-5'-OH formations were inhibited by 21 and 14%, respectively, whereas the influences of anti-CYP2D6 and anti-CYP1A were slight (Table 7).
DISCUSSION

Thalidomide was biotransformed into 5-OH and 5'-OH by human and animal liver microsomes. Recombinant human CYP2C19 and rat CYP2C6 isoforms were primarily responsible for forming these metabolites, and the male rat-specific CYP2C11 formed only 5'-OH. The 5-OH was subsequently hydroxylated to 5,6-dOH by CYP2C19, CYP2C9, and CYP1A1 in humans and by CYP2C11, CYP1A1, CYP2C6, and CYP2C12 in rats. A nonlinear increase of the 5-OH formation in liver microsomes would be attributable to the subsequent metabolism of 5-OH into 5,6-dOH in addition to the spontaneous hydrolysis of the metabolite. Because the male rat-specific CYP2C11 isoform has extensive activities in both cis-5'-OH and 5,6-dOH formations, it is reasonable to assume that male rats could form these metabolites at a greater rate than females. In contrast, the 5-OH formation was slightly greater in female rats than males, which reflects less activity of the subsequent 6-hydroxylation of 5-OH in females. Phenytoin received attention for its toxicity, including teratogenicity, possibly via a reactive intermediate. Interestingly, phenytoin is also a substrate of rat CYP2C6 and CYP2C11, resulting in the sex differences in the metabolite formations (36), whereas the drug is metabolized by CYP2C19 (minor) as well as CYP2C9 (major) in humans. Thus, there appear to be partially common mechanisms of the metabolic activation between phenytoin and thalidomide.

The recombinant CYP2B6 formed lesser amounts of cis-

5'-OH (Table 5). Because the amount of CYP2B6 (<1% of the total CYP) is not more than that of CYP2C19 (around 1%; Refs. 35, 37, 38), the most important CYP isoform for thalidomide metabolism in human liver should be CYP2C19. The tentative $K_m$ of rat microsomes were comparable with those of recombinant CYP2C6 and CYP2C11. The chemical inhibition studies showed that S-mephenytoin and omeprazole, the substrates of CYP2C19, inhibited the metabolite formations by human liver microsomes, supporting the involvement of CYP2C19 in thalidomide metabolism (Table 6). Limited involvement of CYP2C8 and CYP2C9 in the thalidomide metabolism was supported by a lack of the effects by their inhibitors quercetin and sulfaphenazole, respectively. Although the inhibitory effects of these chemicals on the activities of rat CYP isoforms are not generally characterized, the inhibitory effects observed in rat liver microsomes were consistent with those in the recombinant CYP2C6 and CYP2C11.

The contribution of the CYP2C subfamily to thalidomide metabolism in human liver microsomes was also supported by the immunoinhibition of anti-CYP2C antibodies (Table 7). According to the vendor, the microsomes from H112 have higher catalytic activities than those from H42 in S-mephenytoin 4'-hydroxylase (CYP2C19) and diclofenac

### Table 3 Metabolite formations by the induced rat microsomes

<table>
<thead>
<tr>
<th>Agent</th>
<th>Induced for</th>
<th>Total CYP (nmol/mg)</th>
<th>Metabolites (pmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>5-OH</td>
</tr>
<tr>
<td>Saline</td>
<td></td>
<td>0.80</td>
<td>2.25</td>
</tr>
<tr>
<td>Aroclor 1254</td>
<td>CYP1A, -2B</td>
<td>4.00</td>
<td>3.59 (160)</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>CYP2E1</td>
<td>1.13</td>
<td>1.68 (75)</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>CYP3A</td>
<td>1.94</td>
<td>1.79 (80)</td>
</tr>
<tr>
<td>Clotrofenic acid</td>
<td>CYP4A</td>
<td>1.91</td>
<td>3.73 (166)</td>
</tr>
</tbody>
</table>

* The CYP contents were provided from the vendor.
* Results are presented as means of duplicate determinations. Values in parentheses indicate the percentage of the control activities treated with saline.

### Table 4 Metabolite formations by human or animal liver microsomes

<table>
<thead>
<tr>
<th>Agent</th>
<th>Induced for</th>
<th>Total CYP (nmol/mg)</th>
<th>Metabolites (pmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>5-OH</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td>0.43</td>
<td>0.36 (1.0)</td>
</tr>
<tr>
<td>Sprague Dawley rats</td>
<td></td>
<td>0.73</td>
<td>2.16 (6.1)</td>
</tr>
<tr>
<td>Fischer 344 rats</td>
<td>1.22</td>
<td>2.67 (7.5)</td>
<td>10.35 (20.1)</td>
</tr>
<tr>
<td>CD1 mice</td>
<td>1.04</td>
<td>8.26 (23.2)</td>
<td>8.70 (16.9)</td>
</tr>
<tr>
<td>New Zealand rabbits</td>
<td>1.34</td>
<td>2.17 (6.1)</td>
<td>2.49 (4.8)</td>
</tr>
<tr>
<td>Beagle dogs</td>
<td>0.90</td>
<td>4.06 (11.4)</td>
<td>9.81 (19.1)</td>
</tr>
</tbody>
</table>

* The CYP contents were provided from the vendor.
* Results are presented as means of duplicate determinations. Values in parentheses indicate the relative activities compared with the human liver microsomes.

### Table 5 Metabolite formations by recombinant CYP isoforms

<table>
<thead>
<tr>
<th>Isotype</th>
<th>Metabolites (pmol/min/nmol CYP)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5-OH</td>
</tr>
<tr>
<td>Control</td>
<td>&lt;1.6</td>
</tr>
<tr>
<td>Human CYPs</td>
<td></td>
</tr>
<tr>
<td>CYP1A human</td>
<td>&lt;1.6</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>&lt;1.6</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>10.0 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>&lt;1.6</td>
</tr>
<tr>
<td>Rat CYPs</td>
<td></td>
</tr>
<tr>
<td>CYP1A rat</td>
<td>&lt;1.6</td>
</tr>
<tr>
<td>CYP2B1</td>
<td>&lt;1.6</td>
</tr>
<tr>
<td>CYP2C6</td>
<td>18.9 ± 8.1</td>
</tr>
<tr>
<td>CYP2C11</td>
<td>&lt;1.6</td>
</tr>
<tr>
<td>CYP2C12</td>
<td>&lt;1.6</td>
</tr>
<tr>
<td>CYP2D2</td>
<td>7.7 ± 3.8</td>
</tr>
</tbody>
</table>

* Results are presented as means with SD from independent three experiments performed in duplicate.
Table 6  Effects of CYP chemical inhibitors on metabolite formations by human and rat liver microsomes and recombinant enzymes

<table>
<thead>
<tr>
<th>Inhibitors (µM)</th>
<th>S-Mephenytoin (300)</th>
<th>Orphenadrine (100)</th>
<th>Sulfaphenazole (10)</th>
<th>Quercetin (10)</th>
<th>cis-5'-OH \mbox{CYP2C19}</th>
<th>Rat</th>
<th>CYP2C6</th>
<th>H112</th>
<th>cis-5'-OH \mbox{CYP2C19}</th>
<th>Rat</th>
<th>CYP2C6</th>
<th>CYP2C11</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;59</td>
<td>&lt;22</td>
<td>29</td>
<td>23</td>
<td>56</td>
<td>&lt;23</td>
<td>49</td>
<td>33</td>
<td>37</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-Mephenytoin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orphenadrine</td>
<td>59</td>
<td>40</td>
<td>18</td>
<td>ND</td>
<td>65</td>
<td>&lt;23</td>
<td>8</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orphenadrine</td>
<td>95</td>
<td>81</td>
<td>25</td>
<td>15</td>
<td>77</td>
<td>40</td>
<td>28</td>
<td>&lt;3</td>
<td>&lt;6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfaphenazole</td>
<td>105</td>
<td>91</td>
<td>88</td>
<td>105</td>
<td>73</td>
<td>93</td>
<td>89</td>
<td>108</td>
<td>90</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quercetin</td>
<td>23</td>
<td>117</td>
<td>74</td>
<td>108</td>
<td>73</td>
<td>89</td>
<td>82</td>
<td>97</td>
<td>110</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cis-5'-OH</td>
<td>287</td>
<td>54</td>
<td>117</td>
<td>74</td>
<td>108</td>
<td>74</td>
<td>46</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} The 5-OH formations in the control were: 0.33 (pmol/min/mg) by H112, 9.9 (pmol/min/nmol CYP) by CYP2C19, 2.18 (pmol/min/mg) by rat microsomes, and 22.3 (pmol/min/nmol CYP) by CYP2C6 against S-mephenytoin, orphenadrine, sulfaphenazole, and quercetin (dissolved in methanol); and 0.26 by H112, 11.3 by CYP2C19, 2.75 by rat, and 27.4 by CYP2C6 against \( \alpha \)-naphthoflavone (dissolved in DMSO). The cis-5'-OH formations in the control were: 0.80 by H112, 6.4 by CYP2C19, 7.35 by rat, 39.5 by CYP2C6, and 31.9 (pmol/min/nmol CYP) by CYP2C11 against S-mephenytoin, orphenadrine, sulfaphenazole, and quercetin; and 0.56 by H112, 3.9 by CYP2C19, 6.40 by rat, 43.4 by CYP2C6, and 29.5 by CYP2C11 against \( \alpha \)-naphthoflavone.
\textsuperscript{b} Results are presented as means of duplicate determinations. Values indicate the percentage of the control activities with vehicle.
\textsuperscript{c} Microsomes from the individual donor H112 with higher S-mephenytoin 4'-hydroxylation activity than the pooled human liver microsomes. 4'-hydroxylation activity (CYP2C9; 1720 versus 3560 pmol/min/mg) and have lower activities in all other prototype reactions by CYP1A2, -2A6, -2B6, -2C8, -2D6, -2E1, -3A4, and -4A. Thus, the higher metabolic formations by the microsomes having higher CYP2C19 activity agreed with the involvement of the isozyme in thalidomide metabolism. In the experiments using the microsomes from treated rats, the metabolite formations were not increased by the inducers for CYP1A, -2B, -2E, -3A, or -4A, suggesting that these CYP subfamilies are not involved in the main metabolic pathway in rats. Although Aroclor 1254, a commercial preparation of polychlorinated biphenyls, was primarily used as a CYP1A and CYP2B inducer and was expected to increase 5,6-DOH formation from 5-OH, we found a slight decrease of the activity in Aroclor 1254-treated rats. The concomitant suppression of CYP2C11 by Aroclor 1254 would compensate the CYP1A1 activity (39, 40).

Thalidomide undergoes slight CYP-mediated metabolism in this research, substantiating the results found in previous studies, although formations should be underestimated because of the hydrolysis, the subsequent metabolisms, and the epimerization. The lower activity of thalidomide metabolism by humans is attributable to CYP enzymes, not to a lack of cofactors or epoxide hydrolase activity. The recombinant CYP2C19, CYP2C6, and CYP2C11 enzymes were coexpressed with cytochrome \( b_6 \) combined with NADPH-CYP oxidoreductase. In our preliminary experiments, 5-OH and cis-5'-OH formations on the chromatogram did not change with or without the microsomes containing human microsomal epoxide hydrolase (0.4 mg/ml styrene oxide hydrolase activity of 13,830 nmol/min/mg, prepared from a human lymphoblast-expressed cell line; Gentest Corp.). Although mice and rats are generally considered insensitive to thalidomide teratogenesis (17, 18), the microsomes from mice as well as rats could form the metabolites from thalidomide through a CYP system more extensively than in humans and rabbits. This result is rather consistent with previous findings that thalidomide acts as an angiogenesis inhibitor in mice when administered i.p (41). Some previous reports indicated that rat microsomes did not confer pharmacological activity on thalidomide by CYP-mediated metabolism and that thalidomide had no antiangiogenic effects in mice implanted with tumor cells (4, 12, 42). This discrepancy may be explained by the differences in the experimental methods, especially in the strain or pretreatment of the animals (43). A recent study suggested that male rats would be more sensitive to thalidomide in terms of neurobehavioral teratogenicity (44).

The polymorphic enzyme CYP2C19 catalyzes the biotransformation of thalidomide in humans. The interindividual variation of the enzyme activity caused by its polymorphism could affect the efficacy and toxicity of therapeutic agents catalyzed by this enzyme, such as mephenytoin, certain barbiturates, diazepam, imipramine, omeprazole, and proguanil (34, 45). Polymorphism of CYP2C19 has been studied extensively since the first report on poor metabolizers of mephenytoin, and the frequencies reported were 2–5% in Caucasians and 13–23% in...
Asian populations (45). Because of very low amounts of biotransformation to the metabolites from thalidomide, the contribution of the polymorphic metabolism to the total pharmacokinetics of parent thalidomide would be insignificant. However, the polymorphic activity to form the active metabolites and/or intermediates could segregate the patient population into subgroups that differ in their metabolic ability. The biotransformation would be impaired in poor metabolizers, resulting in very low or absent concentrations of these metabolites. If the clinical impact of the CYP2C19 polymorphism on thalidomide treatment were revealed, a poor metabolizer might require relatively large doses of thalidomide for therapeutic purposes; a normal “extensive” metabolizer might have a higher risk of adverse effects. A small foresighted study phenotyped CYP2C19 using mephenytoin as a probe in three patients suffering from thalidomide neuropathy (27). Although the study was far from conclusive, none of the patients were poor metabolizers. CYP1A1 levels are very low in the adult liver, and this enzyme is mainly expressed in extrahepatic tissues such as lung and intestine, where it is highly induced by cigarette smoking and exogenous compounds such as polycyclic aromatic hydrocarbons and 2,3,7,8-tetrachlorodibenzodioxin (16, 46). Increased CYP1A1 activity by exposure to these inducers might modulate pharmacological effects of thalidomide.

It seems unlikely that a CYP system in fetal liver would form the metabolites or intermediates responsible for teratogenicity. The CYP2C expression and 5-mephenytoin 4'-hydroxylase activity (CYP2C9 activity) are significantly lower in human fetal livers than in adults (47). Additionally, CYP3A7, the major isozyme in fetal liver (48), did not form 5-OH or cis-5'-OH at a quantifiable level in this research. Nonetheless, the CYP2C subfamily has been detected recently in extrahepatic tissues such as human endothelial cells and the intestine; the metabolism of thalidomide in the target organ or cells in the fetus remains to be investigated (49, 50).

The significant expression of the CYP enzyme in the human small intestine includes CYP2C19 as well as CYP3A4 and CYP2C9 (50). The polymorphic expression of CYP1A1 is also known in the human small intestine (46). CYP1A1, -2B1, -2C6, -2C11, and -3A1 are all expressed in the small intestine of the rat, and CYP1A1 is the most inducible (51). Large intersubject variability of expression of the thalidomide-metabolizing enzymes, as well as the metabolic activity, would affect the bioavailability of oral thalidomide, which might explain, in part, the low bioavailability of thalidomide in rats (41).

A prototype flavonoid, α-naphthoflavone, simultaneously stimulated the 5-OH formation and inhibited cis-5'-OH formation catalyzed by human liver microsomes (Table 6). Similar stimulation of 5-OH formation was observed when the recombinant CYP3A4 enzyme, not CYP2C19, was used instead of human liver microsomes. Thus, the increase of 5-OH formation was mediated by CYP3A4, despite its inability to form 5-OH without α-naphthoflavone at a quantifiable level. The stimulation of the 5-OH formation in human liver microsomes is underestimated because of the inhibited CYP2C19 activity by α-naphthoflavone. Some flavonoids have been known to stimulate the biotransformation of chemicals, such as benzo[a]pyrene, aflatoxin B1, 17β-estradiol, and carbamazepine, catalyzed by CYP3A4 (52–54). The discrepant effects between the formations of 5-OH and cis-5'-OH are similar to those observed in aflatoxin B1, stimulation in 8,9-epoxidation and inhibition in 3-hydroxylation (54). The mechanism of stimulation of 5-hydroxylation of thalidomide by α-naphthoflavone is unclear; however, the finding implies that 5-OH formation could be increased by dietary flavonoids contained in fruits, vegetables, and grain products.

We discovered definitively that the polymorphic enzyme CYP2C19 is responsible for 5- and 5'-hydroxylation of thalidomide in humans. The 5-OH formation could be environmentally modulated by dietary flavonoid. In rats, thalidomide was hydroxylated extensively by CYP2C6 as well as the sex-specific enzyme CYP2C11. Although there is in vitro evidence that thalidomide requires metabolic activation by CYP, clinical significance of CYP-mediated metabolism of the drug needs to be further evaluated in future clinical studies. We are in the process of clinical research to see whether polymorphism of the CYP2C19 gene is associated with the pharmacokinetics of the metabolites and clinical outcomes of thalidomide. Additionally, when a patient’s genetic markers, including the CYP2C19 genotype, are related to the therapeutic effects of thalidomide, the pharmacogenetic assessment of a patient would be imperative before the administration of thalidomide.

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REFERENCES

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1972 Thalidomide Metabolism by the CYP2C Subfamily


