In Vitro Metabolic Study of Temsiroliimus: Preparation, Isolation, and Identification of the Metabolites

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Received January 29, 2007; accepted May 30, 2007

ABSTRACT:

The in vitro metabolism of temsiroliimus, (rapamycin-42-[2,2-bis-(hydroxymethyl)]-propionate), an antineoplastic agent, was studied using human liver microsomes as well as recombinant human cytochrome P450s, namely CYP3A4, 1A2, 2A6, 2C8, 2C9, 2C19, and 2E1. Fifteen metabolites were detected by liquid chromatography (LC)-tandem mass spectrometry (MS/MS or MS/MS/MS). CYP3A4 was identified as the main enzyme responsible for the metabolism of the compound. Incubation of temsiroliimus with recombinant CYP3A4 produced most of the metabolites detected from incubation with human liver microsomes, which was used for large-scale preparation of the metabolites. By silica gel chromatography followed by semipreparative reverse-phase high-performance liquid chromatography, individual metabolites were separated and purified for structural elucidation and bioactivity studies. The minor metabolites (peaks 1–7) were identified as hydroxylated or desmethylated macrolide ring-opened temsiroliimus derivatives by both positive and negative mass spectrometry (MS) and MS/MS spectroscopic methods. Because these compounds were unstable and only present in trace amounts, no further investigations were conducted. Six major metabolites were identified as 3-hydroxyl temsiroliimus (M8), 35-hydroxyl temsiroliimus (M9), 11-hydroxyl temsiroliimus with an opened hemiketal ring (M10 and M11), N-oxide temsiroliimus (M12), and 32-O-desmethyl temsiroliimus (M13) using combined LC-MS, MS/MS, MS/MS/MS, and NMR techniques. Compared with the parent compound, these metabolites showed dramatically decreased activity against LNCaP cell proliferation.

Materials and Methods

Chemicals and Reagents. Temsiroliimus and recombinant cytochrome P450s (3A4, 1A2, 2A6, 2C8, 2C9, and 2E1) were obtained from the Bioprocess Department of Wyeth Research (Pearl River, NY). Human liver microsomes were purchased from BD Biosciences (Woburn, MA). Reagents for the NADPH generation system (disodium salt of NADP, t-glucose 6-phosphate, and glucose-6-phosphate dehydrogenase) were purchased from Sigma-Aldrich (St. Louis, MO). Other reagent-grade chemicals and HPLC-grade solvents were purchased from EM Science (Gibbstown, NJ) or J. T. Baker (Phillipsburg, NJ).

Incubation of Temsiroliimus with Human Liver Microsomes. Temsiroliimus (50 μM) was incubated with 1 mg/ml human liver microsomal proteins in 0.1 M phosphate buffer solution (pH 7.4) containing 1 mM EDTA and 4 mM MgCl2. Reactions were initiated by addition of the NADPH-regenerating system, resulting in a final concentration of 4 mM glucose 6-phosphate, 2 mM NADP, and 1 unit/ml glucose-6-phosphate dehydrogenase. The incubations were carried out at 37°C in a shaking water bath. Control incubations without the NADPH-regenerating system were performed under the same conditions. The reactions were terminated by the addition of cold acetonitrile. Precipitated materials were removed by centrifugation at 5000 rpm for 10 min at 4°C, and the supernatants were collected and dried under vacuum. The dried extracts containing the metabolites were reconstituted in acetonitrile-water (7:3) for HPLC and LC-MS analysis.

Incubation of Temsiroliimus with Recombinant Human P450s. Selected recombinant P450s (3A4, 1A2, 2A6, 2C8, 2C9, and 2E1; 0.3 nmol each) were individually incubated with temsiroliimus (50 μM). The other components of...
the reaction mixtures were the same as above for the incubation of human liver microsomes. After incubation for 40 min at 37°C, the reactions were quenched with cold acetonitrile. Subsequent sample treatment was the same as for the human liver microsome samples.

**Chemical Inhibition of P450 3A4 Activities.** Ketoconazole in ethanol (10 µl) was added to each tube containing the human liver microsomes (1 mg/ml), EDTA (1 mM), MgCl₂ (4 mM), temsirolimus (50 µM), and 0.1 M potassium phosphate buffer (pH 7.4), giving a total reaction volume of 900 µl. The final concentration of ketoconazole in the assays ranged from 1 to 100 µM. Duplicate samples were prepared at each concentration. The samples were preincubated for 2 min at 37°C, and then 100 µl of an NADPH-regenerating system (same as described above) was added to initiate the reactions. The reactions were incubated at 37°C for 15 min and then quenched with cold acetonitrile. Subsequent sample processing was the same as described above.

**Large-Scale Preparation of Temsirolimus Metabolites.** The large-scale incubation was conducted in a 5-liter reactor containing temsirolimus (400 mg in 10 ml of ethanol), recombinant CYP3A4 (1200 nmol), MgCl₂ (400 mM × 40 ml), and the NADPH-regenerating system (final concentration: 4 mM glucose 6-phosphate, 1.6 mM NADP, and 0.6 unit/ml glucose-6-phosphate dehydrogenase) in 0.1 M potassium phosphate buffer for a total volume of 4 liters. The incubation was conducted under oxygen (bubbled O₂ at 0.30 liter/min) with agitation at 125 rpm. After incubation for 60 min at 37°C, the mixtures were cooled to 25°C and then extracted twice with equivalent volumes of ethyl acetate. The ethyl acetate extracts were combined after the solvent was evaporated under vacuum to yield approximately 1 g of crude extract.

**Isolation of the Metabolites from Large-Scale Preparations.** The crude extract was dissolved in 4 ml of acetone and loaded onto a silica gel flash column. The column was sequentially eluted with hexane/acetone and acetone/methanol gradients. A total of 20 fractions were collected (350 ml/fraction) and analyzed by LC-MS. Fractions 1 to 9 consisted mainly of lipids from the CYP3A4 membranes (176 mg), fractions 9 to 12 contained unmodified temsirolimus (290 mg), fractions 13 to 17 contained the temsirolimus metabolites (96 mg), and fractions 18 to 20 contained the polar pigments from the membranes (145 mg). Fractions 13 to 17 were combined for further separation by semipreparative HPLC on a Supelcosil LC-C18 column (10 × 250 mm, 5 µm). A stepwise gradient consisting of methanol/water with 5 mM ammonium acetate was used at a flow rate of 2.0 ml/min (methanol from 60 to 70% in 30 min and from 70 to 82% in 60 min). Separation was monitored by a UV detector at both 220 and 280 nm. Each metabolite was collected in a separate container on ice as it eluted from the HPLC column. After the organic solvents were removed by vacuum evaporation, the aqueous residues were lyophilized to provide the purified individual metabolites.

**HPLC, LC-MS, MS/MS, and NMR Methods.** Analytical HPLC was

Fig. 1. Representative HPLC chromatograms of temsirolimus incubation products in (top) HLM control, (1b) HLM/NADPH-regenerating system, (1c) recombinant 3A4/NADPH-regenerating system; and (1d) recombinant 3A4 control. AU, arbitrary units.
CID energy was collision energy was 30 V or lower (the molecular ion was not detectable if the macrolide ring-opened molecules (seco-temsirolimus type derivatives), the structures. For temsirolimus and analogs, the collision energy was 50 V, but

Electrospray ionization was conducted in both negative and positive modes. Tandem mass spectrometer coupled with a Shimadzu-10Advp HPLC system. Applied Biosystems-PE Sciex QSTAR PULSAR quadrupole time-of-flight

step of 0.2. The negative ion mode was recorded. (ESI) mass spectra were acquired at unit resolution from scan atmospheric pressure chemical ionization and electrospray ionization analysis, and half of the solvent was channeled into the mass spectrometer. Full

metabolism calculation. MS/MS fragment ions and their corresponding elemental compositions were calculated using the measured molecular ion and a common known fragment ion as references. The LC-NMR data of M8 were acquired on a 600-MHz Bruker DRX spectrometer equipped with a single flow cell LC-NMR probe. The LC separation was performed on an X Terra column (4.6 × 250 mm, 5 μm) with a mobile phase of D2O and acetonitrile-d3, (acetonitrile-d3 from 50 to 90% in 20 min at a flow rate of 0.6 ml/min). Proton spectra were collected with double-solvent suppression. For the other metabolites, the 1H NMR data were acquired on a 500-MHz Bruker DRX 500 spectrometer in acetonitrile-d3.

Results

Temsriolimus Metabolism with Human Liver Microsomes and Recombinant CYP3A4. Incubation of temsirolimus with human liver microsomes in the presence of the NADPH-regenerating system indicated that the total metabolite concentration reached a maximum after 40 min. At that time, the concentration of temsirolimus was reduced by 22 ± 3%, which included its conversion to the macrolide ring opened product, seco-temsirolimus (12 ± 3%) (Fig. 1). A representative HPLC chromatogram of the metabolites generated by human liver microsomes is shown in Fig. 1b. The major peak at 25.6 min, which is also observed in the control samples, is seco-temsirolimus identified by direct LC-MS comparison with the authentic standard. Because it was also present in the control samples, this compound was considered to be a nonspecific degradation product that resulted from a hydrolysis of the macrocyclic lactone ring followed by dehydration of C25/C26. The same ring-opening product, seco-rapamycin, had also been detected from the incubation of rapamycin with human liver microsomes and the pooled bile of intravenously dosed rats (Wang et al., 1997). As for seco-rapamycin, seco-temsirolimus did not show any antitumor and FKBP binding activities due to the macrolide ring opening. Compared with the control incubation (Fig. 1a), 15 new peaks appeared in the human liver microsome reaction. Peaks 1 to 7, peak 11', and peak 14 were the minor products, observed at trace levels (each peak area was <0.3% of the total HPLC area of the analyzed sample). Peaks 8 (2.6%), 9 (0.43%), 10 (0.68%), 11 (0.82%), 12 (0.40%), and 13 (0.88%) were the major metabolites designated as M8 through M13 for convenience. The formation of these new peaks was inhibited by bubbling CO through the reaction solution during incubation (data not shown). This observation suggested that biotransformation of temsirolimus required the presence of microsomal P450 monoxygenases.

To identify the enzymes involved in biotransformation, temsirolimus was individually incubated with each of six recombinant

![Fig. 2. Expanded semipreparative HPLC chromatograms of temsirolimus incubation products in HLM/NADPH (top) and in recombinant CYP3A4/NADPH (bottom).](image-url)
human P450 enzymes: 1A2, 2A6, 3A4, 2C8, 2C9, and 2E1. Experimental results indicated that recombinant CYP3A4 generated almost all of the metabolites detected in the human liver microsome studies, with the exception of peak 11', which is shown in Fig. 1c. No significant metabolism was noted in any of the other recombinant P450 reactions.

Further comparison of the metabolic products produced by human liver microsomes and by recombinant CYP3A4 was also performed by LC-MS. After the metabolites were separated and collected from semipreparative HPLC, each individual metabolite peak was analyzed by LC-MS. The semipreparative HPLC chromatograms of the metabolic products from human liver microsomes and from incubation with recombinant CYP3A4 are shown in Fig. 2, and the LC-MS results are summarized in Table 1. The major metabolites (M8–M13) produced in human liver microsomes and in recombinant CYP3A4 incubations displayed closely similar retention times and the same molecular ions.

**Inhibition of Temsirolimus Metabolism.** Ketoconazole, an antifungal drug, is a potent inhibitor of CYP3A4 (Maurice et al., 1992). The addition of ketoconazole to the reaction inhibited the biotransformation of temsirolimus by human liver microsomes. The effect of ketoconazole on the formation of the major metabolites is shown in Fig. 3. Ketoconazole inhibited the formation of metabolites M8 through M13 with an IC₅₀ of 2 µM. The addition of 5 µM ketoconazole decreased metabolite formation to 10 to 20% of control levels. This result confirmed the fact that CYP3A4 was the main enzyme responsible for the biotransformation of temsirolimus.

**Isolation and Structure Elucidation of Temsirolimus Metabolites.** To obtain enough material for structural elucidation and antitumor activity studies, a 4-liter reaction was performed by incubating 400 mg of temsirolimus with 1200 nmol of CYP3A4 and the NADPH-regenerating system. After solvent extraction, the crude ex-
TABLE 2

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>[M-H]⁻ m/z</th>
<th>Fragment Ions, m/z</th>
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<tr>
<td>Temsirolimus</td>
<td>1028.7</td>
<td>437, 407, 389, 371</td>
</tr>
<tr>
<td>M8</td>
<td>1044.6</td>
<td>437, 407, 389, 371</td>
</tr>
<tr>
<td>M9</td>
<td>1044.6</td>
<td>437, 407, 389, 371</td>
</tr>
<tr>
<td>M10</td>
<td>1044.6</td>
<td>437, 407, 389, 371</td>
</tr>
<tr>
<td>M11</td>
<td>1044.6</td>
<td>437, 407, 389, 371</td>
</tr>
<tr>
<td>M12</td>
<td>1044.6</td>
<td>437, 407, 389, 371</td>
</tr>
<tr>
<td>M13</td>
<td>1014.6</td>
<td>437, 407, 389, 371</td>
</tr>
</tbody>
</table>

Numbers in boldface are the m/z values of key fragment ions used in structural elucidation.

Fig. 5. a, proposed structure of M8 and the fragmentation assignments (observed fragment ions are listed in Table 2). b, an expanded ¹H NMR spectrum of M8 compared with that of temsirolimus.
tract was subjected to silica gel fractionation to eliminate the unre-
acted starting compound and the impurities from CYP3A4 mem-
branes. The crude metabolite mixture was then separated by
semi-preparative HPLC to yield purified individual metabolites for
structure elucidation.

The minor components appearing before seco-temsirolimus in the
preparative HPLC chromatogram (peaks 1–6) (Fig. 2) were either
hydroxylated or desmethylated temsirolimus derivatives, as suggested
by their molecular ions listed in Table 1. For example, the peak with
[M-H]⁻ at m/z 1060 was a dihydroxylated temsirolimus derivative,
the peak with [M-H]⁻ at m/z 1044 was a monohydroxylated temsi-
rolimus derivative, and the peak with [M-H]⁻ at m/z 1046 was either
desmethyl-dihydroxyl temsirolimus or a temsirolimus ester
bond hydrolysis product. As for seco-temsirolimus, these components
easily fragmented in the positive MS/MS/CID mode (i.e., fragmenta-
tion at lower collision energy, ≤30 V), suggesting that they were
macrolide ring-opened derivatives (open at the O24/C25 bond). Peak 7
from both a human liver microsome incubation and a recombinant
CYP3A44 incubation exhibited [M-H]⁻ at m/z 1030 and was identi-
cified as a C37 ketone reduced seco-temsirolimus (C=O converted to
CH–OH) by its MS/MS and MS/MS/MS spectra. Considering that
these compounds were unstable, their abundances were very low, and
no biological activity was due to the ring opening, further structural
study was not performed on them. Structure elucidation by MS/MS,
MS/MS/MS, and NMR focused on the major metabolites: M8, M9,
M10, M11, M12, and M13.

**MS/MS Fragmentation of Temsirolimus.** Temsirolimus exhib-
itated a molecular ion at m/z 1028 in the negative ESI MS spectrum,
gave two fragment ions at m/z 590 and m/z 437 in the MS/MS/MS/
CID experiment. These two ions resulted from cleavage of the C31/C32
bond and the O24/C25 ester bond, representing the "southern"
fragment ion of the molecule as suggested by the intact northern fragment ions at
m/z 1044. These fragment ions were used as the diagnostic criteria
for structural elucidation of the metabolites by the mass shift tech-
nique. For example, the presence of the m/z 437 fragment ion in a
metabolite indicated that the northern portion of the molecule was
intact. The disappearance of the group 2 fragment ions at m/z 251, m/z
229, and m/z 147 indicated that biotransformation had occurred in the
C11 to C4 region and/or the C36 position of the southern portion. Using
this technique, the locations of biotransformation were determined.
Table 2 lists the fragment ions observed in the MS/MS/CID spectra of
temsirolimus and its metabolites.

**Identification of M8.** M8 showed a deprotonated molecular ion at
m/z 1044 in the ESI MS spectrum, 16 Da higher than that of temsi-
rolimus, indicating a monohydroxylation (or oxidation) product of
temsirolimus. The hydroxylation occurred at the southern part of the
molecule as suggested by the intact northern fragment ions at m/z 437
and the modified southern fragment ion at m/z 606 in MS/MS spec-
trum (Table 2). Compared with the parent compound, which showed
the group 2 ions at m/z 251, m/z 229, m/z 147, and m/z 101, M8 gave
the fragment ions at m/z 277, m/z 245, m/z 163, and m/z 101 (Table 2),
suggesting that hydroxylation had occurred on the right side of the
southern part, of either C36 or the C1–C3 moiety (Fig. 5a). Because M8
displayed the same UV absorption as temsirolimus, the triene
group (C1–C3) should be unchanged, thus limiting the possible bio-

**TABLE 3**

| Measured m/z⁴ | Calculated m/z | Elemental Composition | Error 
<table>
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<tr>
<td>504.2618</td>
<td>504.2603</td>
<td>C24H22NO8</td>
<td>+1.5</td>
</tr>
<tr>
<td>472.2351</td>
<td>472.2341</td>
<td>C20H15NO6</td>
<td>+1.0</td>
</tr>
<tr>
<td>175.112</td>
<td>175.1128</td>
<td>C6H6O</td>
<td>-1.6</td>
</tr>
</tbody>
</table>

*⁴ Accurate mass measurements were made using the molecular ion of m/z 1044.5897 and the known fragment ion of m/z 168.0661 as references.
transformation positions to C7, C9, C36, and two methyl groups. M8 was finally identified as 36-hydroxy temsirolimus by LC-NMR. In the 1H NMR spectrum of M8 (Fig. 5b), the disappearance of H16 resonance and the significant downfield shifts of H1 (δ 5.66 ppm) compared with temsirolimus (δ 5.46 ppm) suggested a 36-hydroxylation structure for M8. Because hydroxylation would eliminate H36, the C36-methyl proton of M8 would present as the observed singlet at δ 1.22 ppm instead of a doublet at δ 0.89 ppm in 1H NMR of temsirolimus, corroborating the proposed structure of M8.

**Identification of M9.** M9 also showed a deprotonated [M−H]− at m/z 1044 and the fragment ions at m/z 437 and m/z 606 in the negative ESI mass spectrum, suggesting a southern hydroxylation derivative of temsirolimus. In MS/MS/CID experiment, M9 displayed the same ESI mass spectrum, suggesting a southern hydroxylation derivative of temsirolimus, as suggested by the appearance of C19/C23 had been left intact. The appearance of unique ions at m/z 101 due to the cleavage of C7-C13 region from C1 to C8 and C36 to C32 (Fig. 7). The observation of the group 3 ions at m/z 168 (due to the cleavage of C19/C15 and C23/O24) and m/z 128 (due to the cleavage of the amide bond in the southern part) indicated that the upper left region from C13 to O24 of the southern part was also intact. The appearance of unique ions at m/z 196, m/z 240, m/z 289, and m/z 321 (Table 2) indicated that the hemiketal ring opened and the most likely hydroxylation position was C14. The hydroxylation of C14 led to C14/C15 cleavage in the southern portion to form the highest intensity fragment ion at m/z 240 (Fig. 7). Further loss of CO2 gave rise to the fragment at m/z 196. C14 hydroxylation also facilitated the cleavage of the C6/C16 bond, giving the fragment ion at m/z 321, which went on to lose MeOH for a strong peak at m/z 289 (Fig. 7). The same fragmentation pattern had also been observed in the MS/MS/CID spectrum of 11-hydroxyl rapamycin from a previous study we had conducted. For M10, the hemiketal ring opening increased the flexibility of the macrolide. The present of more conformation isomer and tautomer forms in solution led its 1H NMR spectrum to become too complicated to be interpreted. To confirm the structure, accurate mass measurements and elemental composition analysis of the diagnostic fragments (m/z 321, m/z 289, m/z 240, and m/z 196) were performed (Table 4), which provided the support for this structure assignment.

**Identification of M12.** M12 was also an oxidative metabolite in the southern region, as indicated by its molecular ion signal at m/z 1044 and the southern fragment ions at m/z 606, m/z 588, m/z 562, m/z 544, m/z 530, and m/z 512 (Table 2; Fig. 8a). The presence of the same group 2 ions (m/z 261, m/z 229, m/z 147, and m/z 101) (Table 2) as for temsirolimus in the MS/MS/CID spectra. The presence of the opened hemiketal ring ions (m/z 240 and m/z 196) suggested that M11 has the same structure as M10, and was, most likely, an epimer of M10 with a different stereochemistry at C14 (Fig. 7).
C₈H₁₀NO₄ and C₆H₁₀NO₃ (based on accurate mass measurements), respectively, pointed to oxidation at the piperidinyl ring (Fig. 8a). In the MS/MS/MS spectra, these two ions generated the fragment ions at m/z 168 and m/z 128, respectively, by eliminating oxygen, but not m/z 166 and m/z 126 from losing water, indicating that M12 was an N-oxide derivative of temsirolimus (Fig. 8a). This result was supported by the observation of the downfield shifts of H₂₂ (δ 5.25 ppm) and H₁₈ (δ 3.65 ppm) compared with those of the parent compound (H₂₂: δ 5.12 ppm, overlapping with H₂₅) observed in the ¹H NMR spectra (Fig. 8b).

Identification of M13. In the negative ESI mass spectrum, M13 showed a molecular ion at m/z 1014 and fragmentation ions at m/z 437 and m/z 576, corresponding to a southern desmethylation derivative of temsirolimus (Table 2). The MS/MS spectrum of the molecular ion gave the same group 3 ions, but different group 2 ions compared with temsirolimus (Table 2), suggesting that the C₉ to O₂₄ region remained unchanged, and biotransformation had occurred in the lower right region of the southern part. The most likely desmethylation candidates were C₇ and C₃₂ (Fig. 9a). The presence of the fragment ion at m/z 147, corresponding to the cleavage of C₈/C₉ and C₃₅/C₃₆ in the southern part, suggested that the C₇-O-methyl group was still intact and that C₃₂-O-desmethylation had occurred. This suggestion was supported by the disappearance of the fragment ion at m/z 101, and the appearance of the ion peak at m/z 87 from C₃₄/C₃₅ cleavage of the southern part. Therefore, the structure of M13 was proposed as C₃₂-O-desmethyl temsirolimus (Fig. 9a). In the ¹H NMR spectrum of M13, the disappearance of C₃₅-methoxyl proton signals at δ 3.24 and the downfield shifting of H₁₁ compared with temsirolimus further confirmed this assigned structure (Fig. 9b).

Biological Activity of the Major Metabolites. The biological activities of rapamycin and temsirolimus are dependent on the binding of the left-hand portion (C₈-C₃₁, the “binding domain”) of the molecule to FKBP12 to form a complex that in turn binds the mTOR protein through the remaining portion of the molecule (the “effector domain”) (Odagaki et al., 1997; Sedrani et al., 1999). The inhibition of the mTOR pathway by FKBP12-rapamycin (or temsirolimus) blocks multiple downstream signals and leads to a general antiproliferative effect. Therefore, any structural modification of the macrocyclic lactone ring (such as ring opening or hydroxylation of these two do-
mains) could potentially affect the antineoplastic activities of the molecule. According to our proposed structures, the biotransformation locations of these metabolites were either in the binding domain (M10, M11, and M12), or in the effector domain (M8, M9, and M13); therefore, antitumor activities different from those of temsirolimus were expected. The antitumor activity of the metabolites was evaluated in a cellular assay of LNCaP (a prostate carcinoma cell line) proliferation. Compared with temsirolimus, which showed an IC₅₀ of 2 nM, these metabolites showed remarkably decreased activity (IC₅₀ > 100 nM) or absolutely no inhibitory activity (IC₅₀ > 1000 nM), as seen in Table 5.

**Discussion**

Temsirolimus, an ester derivative of rapamycin, is a selective inhibitor of mTOR that showed clear clinical efficacy and excellent tolerability in the treatment of renal cancer. The in vitro metabolism studies conducted with temsirolimus and human liver microsomes led to the formation of 15 metabolites as detected by LC-MS. As with rapamycin, CYP3A4 was shown to be the major enzyme responsible for biotransformation through an inhibition study and by incubation with individual recombinant P450 enzymes. Such knowledge is of considerable clinical utility with regard to potential drug-drug interactions, as well as interindividual differences in drug-metabolizing capacities stemming from genetic polymorphisms, because CYP3A4 is involved in the biotransformation of approximately 60% of all the xenobiotics on the market today (Waxman et al., 1988; Guengerich, 1989; Thummel and Wilkinson, 1998), including cyclosporine, erythromycin, diazepam, nifedipine, estradiol, paclitaxel, and lovastatin. Coadministration of drugs that are substrates (such as erythromycin, clarithromycin, and lovastatin), inhibitors (such as troleandomycin and human immunodeficiency virus protease inhibitors), or inducers

**TABLE 5**

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC₅₀ (nM)</th>
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<tbody>
<tr>
<td>Temsirolimus</td>
<td>2</td>
</tr>
<tr>
<td>Seco-temsirolimus</td>
<td>~5000</td>
</tr>
<tr>
<td>M8</td>
<td>~1000</td>
</tr>
<tr>
<td>M9</td>
<td>~100</td>
</tr>
<tr>
<td>M10</td>
<td>~200</td>
</tr>
<tr>
<td>M11</td>
<td>~200</td>
</tr>
<tr>
<td>M12</td>
<td>~1000</td>
</tr>
<tr>
<td>M13</td>
<td>~1000</td>
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</table>
(such as rifampin and carbamazepine) of CYP3A4 may affect the metabolic disposition of temsirolimus in humans. An earlier study on the effects of temsirolimus on patients with recurrent glioblastoma multiforme (Galanis et al., 2005), who were at the same time receiving P450-inducing anticonvulsants (known to increase CYP3A4), showed that the peak concentrations (C_{\text{max}}) of temsirolimus and rapamycin decreased by approximately 73 and 47%, respectively, compared with those in patients with renal cancer who were not receiving P450-inducing anticonvulsants.

The 15 detectable metabolites were the monohydroxylation, desmethylation, N-oxidation, and dihydroxylation/ring-opening products of temsirolimus. Six major metabolites were identified to be 36-hydroxyl temsirolimus (M8), 35-hydroxyl temsirolimus (M9), 11-hydroxyl temsirolimus with an opened hemiketal ring (M10 and M11), N-oxide temsirolimus (M12), and 32-O-desmethyl temsirolimus (M13) by combined LC-MS, MS/MS, MS/MS/MS, and NMR spectroscopic methods. In humans, temsirolimus converts by hydrolysis to rapamycin, and both temsirolimus and rapamycin are subject to oxidative metabolism (Galanis et al., 2005). However, no rapamycin metabolites were observed in our study, most likely because the formation of rapamycin from temsirolimus in human liver microsomes was not extensive. Temsirolimus metabolites formed primarily via C-oxidation (aliphatic hydroxylation), O-desmethylation, N-oxidation, and ring opening, which was similar to the metabolites found in pooled bile of rats after they were administered rapamycin intravenously (Wang et al., 1997). In addition to seco-rapamycin, nine hydroxylated rapamycin and/or desmethylated rapamycin metabolites were detected in the same pooled rat bile. Although the structures of these metabolites were not identified, the hydroxylation and desmethylation sites of three major biliary metabolites (m2, m10, and m13) were proposed at the regions of C32 to C36 and C1 to C11 on the basis of LC-MS data, resembling temsirolimus metabolites M8, M9, M10, and M11. Two southern portion monohydroxylated rapamycin products were also found as the most abundant metabolites (~73.6% of total metabolites) from the blood of kidney transplantation patients receiving rapamycin (Holt et al., 2003). Whether the temsirolimus metabolites identified in our study share the same biotransformation locations with those in vivo rapamycin metabolites need to be further evaluated.

Although the 41-O-desmethylated product was the predominant metabolite of rapamycin in the incubation of rapamycin with human liver microsomes, this was not the case for temsirolimus. Such metabolic differences may be a consequence of C_{42}-O-esterification of temsirolimus. Introduction of the 2,2-bis-(hydroxymethyl)-propionate side chain at the C_{42} position resulted in significant steric hindrance, thus blocking CYP3A4 enzyme action on the 41-O-methyl group. It is worth mentioning that the major in vitro rapamycin metabolite, 41-O-desmethyl rapamycin, was not found in rat bile and blood of kidney transplantation patients in significant amounts according to the studies of Wang et al. (1997) and Holt et al. (2003). This result is probably due to the fact that 41-O-desmethyl rapamycin underwent further oxidation and/or ring opening and degraded to various products at very low amounts. It is also possible that rapamycin (or temsirolimus) alters its structure conformation after binding to target proteins in vivo, which changes CYP3A4 action and results in a different bio-transformation profile than that found in vitro.

Incubation of temsirolimus with recombinant human CYP3A4 in the presence of a NADPH-regenerating system produced almost all of the metabolites detected in human liver microsomes, so this technique was subsequently used to prepare the metabolites on a large scale for isolation and analysis of the metabolites. Six major metabolites (M8–M13) were isolated and purified, enabling unambiguous structural elucidation and bioactivity studies and also providing useful reference standards for future in vivo metabolic studies.

As temsirolimus is a relatively complicated molecule, the structural elucidation of the metabolites was challenging. Combined LC-MS, MS/MS, MS/MS/MS, and accurate mass measurements as well as NMR techniques provided powerful and reliable tools for metabolite structural elucidation. Temsirolimus usually is present in solution as two interchangeable isomer forms, and the most proton signals overlapped in the NMR spectrum. For metabolites, hydroxylation and/or ring opening increases the complexity of the proton NMR spectrum. The characteristic fragmentation pattern of temsirolimus in MS/MS and MS/MS/MS and accurate mass measurements of the fragments can provide more structural information for those metabolites (such as M9, M10, and M11) with heavily overlapping and uninterpretable NMR data.

Acknowledgments. We are grateful to Dr. Melissa Lin and Yumin Gong for acquiring NMR data of temsirolimus metabolites.

References


