Similarity of Metabolism for CAI (NSC 609974) in Human Liver Tissue in Vitro and in Humans in Vivo

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ABSTRACT

Metabolism of a new antitumor agent, CAI (NSC 609974), was investigated in human liver tissue in vitro and in plasma and urine of patients receiving CAI. Metabolites identified by HPLC following 14C-labeled CAI incubation with human liver microsomes and reported as percentage of total metabolites formed were M1 (0–3.4%), M2 (4.5–33%), M3 (60–79%), and M4 (7.2–19%). Ketoconazole, an inhibitor of cytochrome P450 3A4, prevented formation of M1, M3, and M4 (concentration of drug that inhibited metabolite formation by 50% when compared to maximum uninhibited activity, >50 µM). CAI incubated with recombinant human P450 3A4 microsomes produced metabolites M3 and M4. Conjugation of M3, most likely a glucuronide, was observed after incubation of 14C-labeled CAI and UDP-glucuronic acid with human liver 13,000 × g supernatant. Plasma samples from patients receiving CAI contained CAI (3.1–5.0 µg/ml), M1 (0.9–2.6 µg/ml), and M2 (1.0–2.2 µg/ml). CAI and M3 but not M4 were observed in the urine samples. After incubation of the urine samples with β-glucuronidase, CAI concentrations increased 67%, M3 increased up to 9-fold, and M4 was detected. CAI is metabolized in vitro and in vivo by both Phase I and Phase II enzymes and is metabolized to M3 and M4 by P450 3A4. These studies suggest that elevated levels of CAI may result from P450 3A4 inhibition by ketoconazole if these two drugs are coadministered. Correlation between CAI metabolism in vitro and results obtained in patients demonstrates the usefulness of liver metabolism studies in vitro in the early stages of drug development.

INTRODUCTION

CAI (NSC 609974), a substituted carboxamido-triazole (Fig. 1), has been developed for clinical trials based on its inhibitory effects on cancer cell proliferation, adhesion, and motility in vitro and in human xenograft models of ovarian cancer and melanoma progression (1, 2). Current studies show the mechanism of action is inhibition of calcium influx-sensitive signal transduction pathways important in the regulation and activation of metastasis and proliferation (2–6). No information concerning CAI metabolism has been reported. However, we observed two significant potential metabolite peaks in the plasma of patients treated with CAI in an ongoing Phase I study at the National Cancer Institute (7). This observation prompted us to investigate the metabolism of this new antitumor agent.

The importance of drug metabolism by microsomal P450 enzymes and the therapeutic and toxic consequences are now well recognized. Drugs that require metabolic activation or are cleared from the body by metabolic processes can have the circulating levels of their pharmacological or toxic species profoundly affected by genetic polymorphism and enzyme inhibition or induction during multiple drug therapy (8–10). The recent isolation and classification of P450 enzymes responsible for drug metabolism have prompted investigators to develop techniques using human liver tissue in vitro to identify the specific P450 enzymes responsible for a drug’s metabolism. The demonstration of the usefulness of these in vitro techniques in the early stages of drug development as tools for probing metabolic pathways and potential drug interactions is of significant interest.

The three major P450s in adult liver involved in drug metabolism belong to families 1, 2, and 3. P450 3A4 varies widely among individuals, accounting for up to 60% of the total P450 present in some liver specimens (10). We studied Phase I metabolism of CAI in vitro by human liver microsome preparations and examined the effect of ketoconazole, a potent cytochrome P450 3A4 inhibitor, on the microsomal metabolism of CAI. To further establish the P450 enzyme partially responsible for the metabolism of CAI, we incubated CAI with rh3A43 microsomes. One of the more common Phase II reactions is the combination of glucuronic acid with various acceptor groups to form the corresponding water-soluble glucuronide conjugate. Phase II metabolism of CAI was investigated by adding UDPGA to incubations of CAI with the S9 fraction of human liver tissue. Plasma and urine samples from patients receiving CAI...
CAI were analyzed using HPLC and these observations were compared to the metabolic profile of CAI in human liver tissue in vitro.

MATERIALS AND METHODS

Human liver specimens, medically unsuitable for transplantation, were acquired under the auspices of the Washington Regional Transplant Consortium (Washington, DC) or the International Institute for the Advancement of Medicine (Exeter, PA). Microsomal protein (rh3A4) from human cells transfected with cDNA-encoded CYP3A4 and control microsomal protein (without rh3A4) were obtained from Gentest (Woburn, MA). 14C-labeled CAI (13.3 mCi/mmol; 31.4 µCi/mg, 99%) was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). Acetonitrile, methanol, and isopropanol were HPLC grade and used as purchased. Other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Preparation of Liver Microsomes. Human liver samples were obtained and immediately sectioned and stored at −80°C until used. Microsomes were prepared by tissue homogenization and differential centrifugation as described by Lake (11). The S9 fraction was obtained by collecting a portion of the supernatant of the tissue homogenate following centrifugation at 13,000 × g for 20 min. The final pellet (microsomes) was resuspended in 10 mM sodium phosphate, with 5 mM MgCl2, and 1 mM EDTA (pH 7.4). Protein concentration of the microsomal fractions was measured using the Bio-Rad (Hercules, CA) method and the fractions were stored at −80°C until used. The metabolic competence of the microsomes has been characterized for the major human drug-metabolizing enzymes.

Metabolism of CAI by Human Liver Microsomes. Preliminary experiments demonstrated that the formation of CAI metabolites by human liver microsomes increased in a linear fashion for incubation times up to 2 h and over substrate concentrations ranging from 12 to 47 µM. Experiments with increasing microsomal protein concentration demonstrated that 1 mg microsomal protein produced the best results. Each incubation mixture contained 1 mg microsomal protein, 14C-labeled CAI, and 10 µl of a NADPH generating system in a 1-ml solution containing: BSA (0.25%), 0.1 mM sodium phosphate buffer (pH 7.4), 1 mM EDTA, and 5 mM MgCl2. 14C-labeled CAI (2.5–3.0 mM, dissolved in ethanol) was added to a final concentration of 25–30 µM. The NADPH generating system consisted of 10 mM glucose-6-phosphate, 1 mM NADP+, 5 mM MgCl2, and 1 unit/ml glucose-6-phosphate dehydrogenase. The BSA was added to limit the precipitation of CAI (a highly lipophilic drug) in the aqueous medium. Following a 2-h incubation at 37°C in a shaking water bath, the reaction was stopped and the protein was precipitated by the addition of 5.0 ml acetone- nitrile. After vortex mixing and centrifugation, 3.0 ml supernatant were dried under vacuum, reconstituted in 100 µl mobile phase, and 25-µl injections were analyzed using HPLC. 14C-labeled CAI added to control samples incubated with heat-inactivated microsomes, without NADPH, and samples incubated for 0 min were analyzed.

Incubations and sample preparation with S9 fractions were performed as described above with substitution of the S9 fraction protein (1 mg) for the microsomal protein (1 mg).

Incubations with microsomes or the S9 fraction were performed in the presence or absence of UDPGA and the results compared. UDPGA was added to a final concentration of 2.3 mM in the incubation mixtures. Fractions of the acetonitrile supernatant from these incubations of CAI with S9 fractions were dried and reconstituted in water containing BSA (0.25%). Duplicates of these reconstituted samples were incubated with and without β-glucuronidase as described below for the incubation and analysis of the urine samples.

Metabolism of CAI by rh3A4 Microsomes. Incubations with rh3A4 microsomes were performed in the same manner as those for the liver microsome incubations. 14C-labeled CAI (17.6 mM, dissolved in methanol) was added to a final concentration of 176 µM and incubated with 1 mg or 2 mg rh3A4 microsomal protein for 1 and 2 h. 14C-labeled CAI (176 µM) was also incubated with control microsomes (2 mg protein) prepared by Gentest from the same cell line without rh3A4 in order to eliminate the possibility of metabolism by enzymes native to the cell line. The incubated samples were prepared and analyzed using HPLC following the procedure for microsomes.

Inhibition of CAI Metabolism in Microsomes. Concentration response inhibition studies with ketoconazole were performed by incubating CAI (28 µM) with human liver microsomes for 2 h in the presence of 0.5–100 µM ketoconazole. The results were compared to samples (maximum uninhibited activity) incubated in the absence of ketoconazole. Ketoconazole vehicle control samples were incubated with 10 µl 0.05 M HCl and analyzed. IC50 values were calculated by fitting the experimental data to an inhibitory sigmoid model with the following equation:

\[ E = E_0 - \frac{E_{max} \cdot C^s}{IC_{50}^s + C^s} \]

using SigmaPlot (Jandel Scientific, San Rafael, CA).

Preparation and Evaluation of Standard Curves. A standard curve in blank plasma was prepared with concentra-
Extraction of Plasma and Urine Samples. Plasma samples from two patients receiving CAl (12) were obtained before treatment and following dosing with CAl for 28 days. A sample following 28 days of dosing was also obtained from a third patient. Extraction of plasma and urine samples were identical, except for preparation of the samples before placement on the extraction column. For plasma, 0.5 ml of sample was added to 1 ml water, mixed, and placed on conditioned 200 mg C18 extraction cartridges (Varian, Harbor City, CA). To 1.5 ml of each urine sample, 0.5 ml 0.5 M phosphate buffer (pH 7.4) was added before placing on the extraction cartridge. Following washes with 4 ml 0.05 M ammonium acetate (pH 6.8) and 4 ml 20% acetonitrile in ammonium acetate, the samples were eluted with 3 ml acetonitrile. The resulting eluants were dried under vacuum, reconstituted in 100 μl mobile phase, and 25 μl were analyzed using HPLC.

HPLC Analysis of CAI and Metabolites. Samples for HPLC analysis were injected on a Hewlett-Packard 1090 HPLC system with UV detection at 263 nm. The separation of CAI and its metabolites was accomplished on an Alltech Adsorbosphere C18 column (3 μm, 4.6 x 100 mm; Alltech, Deerfield, IL). The mobile phase was 50 mM ammonium acetate, acetonitrile, and isopropyl alcohol (60:26:14, v/v) and was pumped at a flow rate of 0.5 ml/min.

For the metabolism studies in vitro containing 14C-labeled CAI, a Radiomatic Flo-Onejeta detector (Packard Instrument Co., Meriden, CT) was connected following the UV detector to monitor the column effluent for radioactivity.

Values reported for metabolites in plasma and urine samples are in terms of CAI equivalents in μg/ml. The UV spectra for CAI, M1, M3, and M4 are very similar. Comparison of relative radioactive peak areas to relative UV peak areas in the microsomal studies indicate that the UV responses for these four compounds were about the same. The spectrum for M2 was different from CAI and the other metabolites. A similar comparison of radioactive to UV peak responses for M2 suggests that a correction factor of approximately 1.9 is needed to report results for this metabolite as CAI equivalents.

RESULTS

CAI Metabolism by Human Liver Fractions. After a 2-h incubation of 14C-labeled CAI (25 μM) with human liver microsomes, at least four radioactive HPLC peaks were observed (Fig. 2A) which were not present in any of the control incubations. The retention times for the metabolites were 7.1

![Image](clincancerres.aacrjournals.org)
Table 1: Metabolism of CAI in microsomes from five human livers and rh3A4 microsomes

<table>
<thead>
<tr>
<th>Microsomal protein</th>
<th>Metabolites (% of total radioactivity)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M1</td>
</tr>
<tr>
<td>HL-IVT</td>
<td>0.41 ± 0.09</td>
</tr>
<tr>
<td>HL-2</td>
<td>0.76 ± 0.09</td>
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<tr>
<td>HL-3</td>
<td>n.d.</td>
</tr>
<tr>
<td>HL-4</td>
<td>n.d.</td>
</tr>
<tr>
<td>HL-9</td>
<td>1.41 ± 0.32</td>
</tr>
<tr>
<td>rh3A4 (1 mg)</td>
<td>n.d.</td>
</tr>
<tr>
<td>rh3A4 (2 mg)</td>
<td>n.d.</td>
</tr>
</tbody>
</table>


**Table 2: Effect of UDPGA on the metabolite M3 and the M3 conjugate in incubations with S9 fractions**

<table>
<thead>
<tr>
<th>Donor code</th>
<th>M3 conjugate</th>
<th>M3</th>
<th>CAI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>UDPGA</td>
<td>Control</td>
</tr>
<tr>
<td>HL-2</td>
<td>1.64 ± 0.30</td>
<td>29.6 ± 1.85</td>
<td>20.9 ± 0.54</td>
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<tr>
<td>HL-4</td>
<td>n.d.</td>
<td>3.51 ± 0.29</td>
<td>2.36 ± 0.34</td>
</tr>
<tr>
<td>HL-9</td>
<td>0.44 ± 0.14</td>
<td>28.0 ± 0.83</td>
<td>19.3 ± 0.49</td>
</tr>
</tbody>
</table>

**Table 3: Effect of β-glucuronidase on the metabolite M3 and the M3 conjugate in S9 fractions incubated with UDPGA**

<table>
<thead>
<tr>
<th>Donor code</th>
<th>M3 conjugate</th>
<th>M3</th>
<th>CAI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Glucuronidase</td>
<td>Control</td>
</tr>
<tr>
<td>HL-2</td>
<td>25.80</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>HL-4</td>
<td>2.44</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>HL-9</td>
<td>23.30</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

**Metabolism of CAI by rh3A4 Microsomes.** Incubation of rh3A4 microsomes with ¹⁴C-labeled CAI resulted in the formation of M3 (Fig. 3). Following a 1-h incubation the amount of M3 produced by 2 mg rh3A4 was 40% more than that produced by 1 mg rh3A4 (Table 1). M4 was observed in both rh3A4 and control incubation samples (Fig. 3), but 2 mg rh3A4 microsomal protein produced approximately three times the amount of M4 observed for 2 mg control microsomal protein.

**Inhibition of CAI Metabolism.** The metabolism of CAI to metabolites M1, M3, and M4 by microsomes was inhibited by ketoconazole (Fig. 4). Ketoconazole IC₅₀ values determined for inhibition of M3 and M4 formation were 2.8 and 3.0 µM, respectively. Although formation of M1 was clearly inhibited at ketoconazole concentrations <2 µM, it was not possible to calculate an IC₅₀ since this metabolite represented only 2% of total CAI metabolism in microsomes. M2 was not inhibited by ketoconazole.
ketoconazole concentrations <5 μM; in fact, the production of M2 appeared to increase under these conditions at the lower ketoconazole concentrations. With the inhibition of the formation of M1, M3, and M4, the concentration of CAI would be higher over the duration of the experiment. More substrate is then available to produce M2. However, at ketoconazole concentrations >50 μM production of M2 was also inhibited. There was no substantial effect on the amount of metabolism in the vehicle control samples when compared to samples with maximum uninhibited activity.

Metabolites of CAI in Human Plasma. Fig. 5A compares plasma samples from one patient before and after 28 days of treatment with CAI. Analysis of three post-dose samples resulted in concentrations of 4.5 μg/ml, 5.0 μg/ml, and 3.1 μg/ml for CAI. Calculation of CAI equivalents in μg/ml for the two metabolites resulted in amounts ranging from 25 to 53% (M1) and 17 to 70% (M2) as percentage relative to the amount of CAI present in each sample.

Metabolites of CAI in Human Urine. Pre-dose and random urine collections obtained 24 h or 28 days following daily or every other day p.o. dosing of CAI were obtained and analyzed using HPLC. Fig. 5B compares a pretreatment urine sample and another sample obtained following dosing with CAI for 28 days. In addition to CAI and M3, a number of significant peaks are observed with retention times between 4 and 10 min which were not present in the pre-dose urine sample. Similar differences in the HPLC chromatograms in the region between 4 and 10 min were observed in two additional patients for which pre- and post-dose urine samples were available for analysis. In addition, random urine collections (blanks) were obtained from five healthy subjects and analyzed. In these samples there were no peaks with responses greater than 20 milli absorbance units between 4 min and 10 min of the chromatographic run.

DISCUSSION

14C-labeled CAI was used to establish the metabolic profile of CAI in human liver preparations. The results of these experiments were then correlated with results from samples obtained from patients receiving CAI during a Phase I trial at the Clinical Pharmacology Branch, National Cancer Institute. Plasma levels in the range of 1–10 μM have recently been reported in this
Fig. 5  CAI and metabolites in human plasma and urine. Chromatograms of plasma (A) and untreated urine (B) samples obtained from subject P2 following daily doses of 500 mg CAI for 28 days (upper traces). Lower traces, corresponding pre-dose samples. Peaks corresponding to M1 and M2 are identified in the plasma. A large peak in the urine, close to M1 in retention time, appears to be composed of more than one component. The inset in B provides a less attenuated view of the chromatogram showing a small peak for M3 and the absence of M4.

The results obtained when UDPGA is added to microsomal incubations of CAI and the results following the further incubation of these samples with β-glucuronidase provide evidence for the formation of a M3 glucuronide conjugate. The rapid conversion of M3, the major microsomal metabolite, to a glucuronide conjugate provides an explanation for the inability to detect M3 in the plasma samples and the low level of M3 found in the untreated urine samples.

Fig. 6  Effect of β-glucuronidase on CAI and metabolites in human urine. Chromatograms for a urine sample from subject P4 obtained 24 h following a single p.o. dose of 540 mg CAI. Following treatment with β-glucuronidase (---), the chromatogram shows an increase in CAI, M3, and M4 when compared to the untreated sample (-----). Inset, an expanded view of the early eluting components in the chromatogram. The peak in the untreated urine is identified as the M3 conjugate. The peak at approximately 7.2 min which increases following treatment with β-glucuronidase is thought to be M1 but cannot be confirmed.

The inhibition profile for metabolite M1, M3, and M4 were similar. The inhibition profile for metabolite M2 was different, suggesting that another cytochrome P450 enzyme is responsible for production of this metabolite. Inhibition of CAI metabolism by ketoconazole, a known inhibitor of P450 3A4, suggested that this enzyme may be primarily responsible for the metabolism of CAI. Further evidence to support this observation was obtained using rh3A4 microsomes, which produced substantial amounts of the major metabolite, M3, plus some M4. Taken together, the metabolism of CAI by rh3A4 microsomes and inhibition of CAI metabolism by ketoconazole at <5 μM (13) suggest that P450 3A4 is involved in the metabolism of CAI. Since these IC50 values are well within ketoconazole therapeutic plasma concentrations (14, 15), it is
Table 4. Results of urine samples from patients receiving CAI

<table>
<thead>
<tr>
<th>Patient</th>
<th>CAI (µg/ml)</th>
<th>M3 (µg/ml)</th>
<th>M4 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>0.19</td>
<td>n.d.</td>
<td>0.44</td>
</tr>
<tr>
<td>P2</td>
<td>1.85</td>
<td>0.11</td>
<td>1.12</td>
</tr>
<tr>
<td>P3</td>
<td>0.45</td>
<td>0.18</td>
<td>1.46</td>
</tr>
<tr>
<td>P4</td>
<td>0.18</td>
<td>n.d.</td>
<td>0.11</td>
</tr>
<tr>
<td>P4d</td>
<td>0.54</td>
<td>0.17</td>
<td>0.72</td>
</tr>
<tr>
<td>P5d</td>
<td>0.30</td>
<td>n.d.</td>
<td>0.26</td>
</tr>
</tbody>
</table>


possible that the concomitant administration of ketoconazole and CAI may result in increased CAI levels.

Our studies demonstrate that CAI is extensively metabolized in *vivo* in human liver tissue and is similar to the metabolic profile observed in humans receiving the drug. Additional studies are warranted to further elucidate the disposition of CAI in humans and to determine the pharmacological activity of metabolites formed.

ACKNOWLEDGMENTS

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