In Vitro Metabolism and Drug-Drug Interaction Potential of UTL-5g, a Novel Chemo- and Radioprotective Agent

Jianmei Wu, Jiajiu Shaw, Sarah Dubaisi, Frederick Valeriote, and Jing Li

Karmanos Cancer Institute, Department of Oncology (J.W., J.L.), and Department of Pharmacology (S.D.), Wayne State University School of Medicine, Detroit, Michigan; 21st Century Therapeutics, Inc., Ferndale, Michigan (J.S.); and Department of Internal Medicine, Henry Ford Health System, Detroit, Michigan (F.V.)

Received July 17, 2014; accepted September 23, 2014

ABSTRACT

N-(2,4-dichlorophenyl)-5-methyl-1,2-oxazole-3-carboxamide (UTL-5g), a potential chemo- and radioprotective agent, acts as a produg requiring bioactivation to the active metabolite 5-methylisoxazole-3-carboxylic acid (ISOX). UTL-5g hydrolysis to ISOX and 2,4-dichloroaniline (DCA) has been identified in porcine and rabbit liver esterases. The purpose of this study was to provide insights on the metabolism and drug interaction potential of UTL-5g in humans. The kinetics of UTL-5g hydrolysis was determined in human liver microsomes (HLM) and recombinant human carboxylesterases (hCE1b and hCE2). The potential of UTL-5g and its metabolites for competitive inhibition and time-dependent inhibition of microsomal cytochrome P450 (P450) was examined in HLM. UTL-5g hydrolysis to ISOX and DCA in HLM were NADPH-independent, with a maximum rate of reaction (Vmax) of 11.1 nmol/min per mg and substrate affinity (Km) of 41.6 μM. Both hCE1b and hCE2 effectively catalyzed UTL-5g hydrolysis, but hCE2 exhibited ~30-fold higher catalytic efficiency (Vmax/Km) than hCE1b. UTL-5g and DCA competitively inhibited microsomal CYP1A2, CYP2B6, and CYP2C19 (IC50 values <50 μM), and exhibited time-dependent inhibition of microsomal CYP1A2 with the inactivation efficiency (kinact/KI) of 0.68 and 0.51 minute−1 mM−1, respectively. ISOX did not inhibit or inactivate any tested microsomal P450. In conclusion, hCE1b and hCE2 play a key role in the bioactivation of UTL-5g. Factors influencing carboxylesterase activities may have a significant impact on the pharmacological and therapeutic effects of UTL-5g. UTL-5g has the potential to inhibit P450-mediated metabolism through competitive inhibition or time-dependent inhibition. Caution is particularly needed for potential drug interactions involving competitive inhibition or time-dependent inhibition of CYP1A2 in the future clinical development of UTL-5g.

Introduction

N-(2,4-dichlorophenyl)-5-methyl-1,2-oxazole-3-carboxamide (UTL-5g) (Fig. 1), a small-molecule tumor necrosis factor-α inhibitor, is under preclinical development as a potential chemo- and radioprotective agent. Although a number of natural and synthetic compounds have been shown to be chemo- or radioprotective, amifostine is the only cytoprotective agent approved by the U.S. Food and Drug Administration for reducing cisplatin-induced cumulative nephrotoxicity and radiation-associated toxic effects on normal oral tissues. Amifostine, as a prodrug, is dephosphorylated by alkaline phosphatase in tissues to a pharmacologically active thiol metabolite that can bind to, and thereby detoxify, reactive metabolites of cisplatin, or scavenge reactive oxygen species generated by exposure to either cisplatin or radiation. The ability of amifostine to differentially protect normal tissues is attributable to the high capillary alkaline phosphatase activity, higher pH, and better vascularity of normal tissues compared with tumor tissue, which results in a more rapid generation and cellular uptake of active thiol metabolites in normal tissues. However, the clinical use of amifostine is limited by its side effects and potential tumor-protective effects (Sadowitz et al., 2002). Thus, there is a compelling need for the development of novel chemo- and radioprotective agents that can reduce the chemotherapy- or radiation-related toxicities while having a good safety profile and little influence on the therapeutic effects of chemo or radiation therapies.

UTL-5g has demonstrated good chemo- and radioprotective activities in preclinical models. Pretreatment of mice with UTL-5g (60 mg/kg, intraperitoneal injection) significantly reduced cisplatin-induced liver, kidney, and hematological toxicities (Shaw et al., 2011). Oral administration of UTL-5g (60 mg/kg) also increased the overall tolerability of high-dose cisplatin, as indicated by increase in survival rate and delayed time to death in mice that were treated with high doses of cisplatin (15 and 20 mg/kg, intravenous injection) (Shaw et al., 2013). In addition, UTL-5g (60 mg/kg, intraperitoneal injection) showed liver protection for acute liver injury induced by radiation, as indicated by decreased elevated levels of aspartate transaminase and alanine transaminase (Shaw et al., 2012). Notably, UTL-5g did not show any tumor-protective effect, but potentiated the antitumor activity of cisplatin in mouse xenograft tumor models (Shaw et al., 2011).

This study was supported by the United States Public Health Service Cancer Center Support [Grant P30 CA024253] and the National Institutes of Health [Grant 2R44-CA141749-02].

dx.doi.org/10.1124/dmd.114.060095.
[This article has supplemental material available at dmd.aspetjournals.org.]

ABBREVIATIONS: DCA, 2,4-dichloroaniline; hCE, human carboxylesterase; HLM, human liver microsomes; ISOX, 5-methylisoxazole-3-carboxylic acid; LC-MS/MS, high-performance liquid chromatography with tandem mass spectrometry; NDGA, nordihydroguaiaretic acid; P450, cytochrome P450; PBS, phosphate-buffered solution; SNP, single nucleotide polymorphism; UTL-5g, N-(2,4-dichlorophenyl)-5-methyl-1,2-oxazole-3-carboxamide.
Although the oral administration of UTL-5g showed excellent chemoprotective activity, its plasma concentrations were below the lower limit of quantitation of the analytical assay after oral administration (60 mg/kg; unpublished data), suggesting that UTL-5g underwent extensive first-pass intestinal and/or hepatic metabolism, and its metabolites were likely pharmacologically active. Further studies confirmed that UTL-5g was a prodrug that required metabolic activation to form the active metabolite 5-methylisoxazole-3-carboxylic acid (ISOX) to exert chemo- and radioprotective activity (Zhang et al., 2014). The hydrolytic conversion of UTL-5g to ISOX and 2,4-dichloroaniline (DCA) (Fig. 1) has been identified in porcine and rabbit liver esterases (Swartz et al., 2013). Nevertheless, little is known about the metabolism of UTL-5g in humans, and the specific enzyme(s) responsible for metabolic activation of UTL-5g has not been defined. Clearly, a better understanding of UTL-5g biotransformation and drug-drug interaction potential will provide important mechanistic insights into the pharmacokinetics and pharmacodynamics of this agent. The obtained information is of great relevance to further rational development into the pharmacokinetics and pharmacodynamics of this agent. The drug interaction potential will provide important mechanistic insights responsible for metabolic activation of UTL-5g has not been defined.

Metabolism and Drug-Drug Interaction Potential of UTL-5g

Table 1: LC-MS/MS parameters for quantitation of UTL-5g and its metabolites (DCA and ISOX)

<table>
<thead>
<tr>
<th>MS mode</th>
<th>Positive</th>
<th>DCA</th>
<th>ISOX</th>
<th>Zileuton (Internal Standard)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS m/z transition</td>
<td>271.17 &gt; 109.96</td>
<td>161.92 &gt; 125.95</td>
<td>128.05 &gt; 109.96</td>
<td>237.08 &gt; 161.03</td>
</tr>
<tr>
<td>Capillary voltage (Kv)</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Cone voltage (V)</td>
<td>20</td>
<td>35</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>Collision energy (Ev)</td>
<td>16</td>
<td>18</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>Desolvation temperature (°C)</td>
<td>350</td>
<td>350</td>
<td>350</td>
<td>350</td>
</tr>
<tr>
<td>Source temperature (°C)</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>Retention time (min)</td>
<td>16.86</td>
<td>14.63</td>
<td>11.85</td>
<td>13.53</td>
</tr>
</tbody>
</table>

*Mobile phase consisted of water (containing 0.1% formic acid) (A) and methanol (containing 0.1% formic acid) (B).
ULTL-5g hydrolysis was examined by co-incubating UTL-5g (40 μM) with NDGA (1–100 μM) in the absence of NADPH at 37°C in a shaking water bath for 10 minutes. Preliminary experiments were conducted to determine the optimal conditions in which linear product formation occurs with respect to hCE1b or hCE2 protein concentration and incubation time. Subsequent experiments were conducted to determine the kinetics of UTL-5g hydrolysis to ISOX and DCA by incubating varying concentrations of UTL-5g (0.5–100 μM) with hCE1b (0.04 mg/ml) or hCE2 (0.01 mg/ml) in 100 mM PBS (pH 7.4) in the absence of NADPH at 37°C in a shaking water bath for 10 minutes.

At the end of the microsomal or enzyme reaction, the reaction (200 μl) was quenched with 800 μl of ice-cold acetonitrile containing 0.1% formic acid and zileuton (1 μM) as the internal standard. The mixture was vortex-mixed for 1 minute and centrifuged at 14,000g at 4°C for 10 minutes, and the supernatant was collected and subjected to high-performance liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis.

**LC-MS/MS Analysis of UTL-5g and Its Metabolites.** UTL-5g and its metabolites (DCA and ISOX) in the supernatants from the HLM or hCE reaction samples were quantitatively determined by a validated LC-MS/MS method using a Waters 2695 high-performance liquid chromatography system coupled with a Waters Quattro Micro triple quadrupole mass spectrometer (Waters, Milford, MA). Chromatographic separation was performed on a Nova-Pak C18 column (4 μm, 3.9 × 150 mm; Waters) at 30°C, running with a gradient mobile phase consisting of water (containing 0.1% formic acid) (A) and methanol (containing 0.1% formic acid) (B) at a flow rate of 0.2 ml/min (Table 1). UTL-5g, DCA, and ISOX were quantitated using multiple reaction monitoring mode. Table 1 shows the mass spectrometric parameters for the analytes. Linear calibration curves for UTL-5g, DCA, and ISOX were constructed in PBS over concentration ranges of 0.02–10 μM. Intra- and interday precisions and accuracies for quality control samples were <15%.

**Assays for Microsomal P450 Activities.** Microsomal P450 activities were assessed by determining the specific metabolic conversions of the known P450 probe substrates in HLM. Specifically, microsomal CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4/5 activities were measured by phenacetin-O-deethylation (acetaminophen), bupropion-hydroxylation, rosiglitazone para-hydroxylation, diclofenac 4'-hydroxylation, S-mephenytoin 4'-hydroxylation, dextromethorphan O-demethylation (dextrophan), and midazolam 1'-hydroxylation in HLM, respectively. The kinetic parameters (V_{max}, K_{m}) for these specific metabolic conversions were determined by incubating varying concentrations of the P450 probe cocktail containing phenacetin (5–320 μM), bupropion (5–320 μM), rosiglitazone (1.25–80 μM), diclofenac (0.625–40 μM), S-mephenytoin (5–320 μM), dextromethorphan (2.5–160 μM), and midazolam (0.625–40 μM) with HLM (0.2 mg/ml) in 100 mM PBS (pH 7.4) in the presence of NADPH-regenerating system (1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 unit/ml glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride) at 37°C in a shaking water bath for 15 minutes.

At the end of the incubation, the reaction (200 μl) was quenched with 800 μl of ice-cold acetonitrile containing the stable isotope-labeled internal standards for individual probe metabolites (Table 2). The mixture was vortex-mixed for 1 minute and centrifuged at 14,000g at 4°C for 10 minutes, and the supernatant was collected and subjected to LC-MS/MS analysis.

**Competitive Inhibition of Microsomal P450 by UTL-5g and Its Metabolites.** The P450 competitive inhibition potential of UTL-5g and its metabolites (DCA and ISOX) was examined by co-incubating varying concentrations of UTL-5g, DCA, or ISOX with the P450 probe cocktail at the probe concentrations equal to their respective K_{i} values, which were determined as shown in Supplemental Fig. 3, in HLM in the presence of NADPH. The reaction mixture (total volume 0.2 ml) contained UTL-5g, DCA, or ISOX (0.5–100 μM), phenacetin (35 μM), bupropion (20 μM), rosiglitazone (5 μM), diclofenac (3 μM), S-mephenytoin (55 μM), dextromethorphan (3 μM), midazolam (2 μM), HLM (0.2 mg/ml), and NADPH-regenerating system (1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 units/ml glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride) in 100 mM PBS (pH 7.4). The reaction was performed at 37°C in a shaking water bath for 15 minutes.

At the end of the incubation, the reaction (200 μl) was quenched with 800 μl of ice-cold methanol containing stable isotope-labeled internal standards for individual probe metabolites (Table 2). The mixture was vortex-mixed for 1 minute and centrifuged at 14,000g at 4°C for 10 minutes. The supernatant was analyzed using the LC-MS/MS assay for the formation of individual probe metabolites, as described earlier.

---

**Table 2**

<table>
<thead>
<tr>
<th>Internal standard (CYP1A2)</th>
<th>Acetaminophen-d₄</th>
<th>OH-bupropion-d₄</th>
<th>5-OH-rosiglitazone-d₄</th>
<th>N/A*</th>
<th>N/A*</th>
<th>N/A*</th>
<th>N/A*</th>
<th>1'-OH-midazolam-d₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS mode</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>N/A*</td>
<td>N/A*</td>
<td>N/A*</td>
<td>N/A*</td>
<td>Positive</td>
</tr>
<tr>
<td>Analyte m/z/transition</td>
<td>150.4 &gt; 109.3</td>
<td>256.22 &gt; 238.23</td>
<td>374.19 &gt; 151.07</td>
<td>N/A*</td>
<td>N/A*</td>
<td>N/A*</td>
<td>N/A*</td>
<td>312.05 &gt; 231.15</td>
</tr>
</tbody>
</table>

*A, N/A, isotope-labeled internal standards were not available. An external standard method was used for quantitation.

**Mobile phase consisted of 10 mM ammonium formate (pH 7.5) (A) and methanol (B).**
Time-Dependent Inhibition of Microsomal P450 by UTL-5g and Its Metabolites. First, the potential for time-dependent inhibition of microsomal P450 by UTL-5g and its metabolites was examined using the P450 probe cocktail. Varying concentrations (5–100 µM) of UTL-5g, DCA, or ISOX were preincubated with HLM (2 mg/ml) in the presence or absence of NADPH-regenerating system in 100 mM PBS (pH 7.4) for 0, 10, 20, 30, and 40 minutes. Ten microliters of preincubation mixture was transferred to the secondary incubation (a total volume of 200 µl) containing the P450 probe cocktail at the probe concentrations equal to 5-fold of their respective Km values (Supplemental Fig. 3) (i.e., 175 µM phenacetin, 100 µM bupropion, 25 µM rosiglitazone, 15 µM diclofenac, 275 µM S-mephenytoin, 15 µM dextromethorphan, and 10 µM midazolam) in 100 mM PBS (pH 7.4) in the presence of NADPH-regenerating system. The secondary incubation was allowed to proceed for 15 minutes at 37°C in a shaking water bath, and the reaction was quenched with 800 µl of ice-cold methanol containing the stable isotope-labeled internal standards. The mixture was vortex-mixed for 1 minute and centrifuged at 14,000 g at 4°C for 10 minutes. The supernatant was analyzed using the LC-MS/MS assay for the formation of individual probe metabolites (Table 2).

The P450 probe cocktail experiment suggested that UTL-5g inhibited microsomal CYP1A2 in a NADPH-, time-, and concentration-dependent manner, while showing insignificant inhibition of other P450 enzymes. Thus, the kinetics for time-dependent inhibition of CYP1A2 by UTL-5g, DCA, or ISOX was further determined. The experiment procedure was the same as the cocktail experiment except for 0.5 mg/ml of HLM used in the primary incubation and only CYP1A2 probe (175 µM of phenacetin) incubated in the secondary incubation.

Positive control experiments with furafylline (a known mechanism-based inhibitor of CYP1A2) were performed by preincubating HLM (0.5 mg/ml) with furafylline (0.5–10 µM) for 0, 10, 20, 30, and 40 minutes followed by measuring the residual CYP1A2 activity (phenacetin-O-deethylation) or residual CYP3A4/5 activity (midazolam 1’-hydroxylation) in the secondary incubation.

Data Analysis. All data analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA). Metabolite formation velocity (v) was calculated as (Cinact/C176 per P450 concentration), where Cinact is the metabolite concentration at the end of incubation and t is the incubation time. The velocity (v) versus initial substrate concentration (Cinact) plots as well as corresponding Eadie-Hofstee (v/Cinact versus Cinact) and Lineweaver-Burk (1/v versus 1/Cinact) plots were constructed. The metabolic kinetic parameters for maximum rate of reaction (Vmax) and substrate concentration at which 50% of the maximum reaction rate is achieved (Km) were estimated by fitting the v versus Cinact data to the Michaelis-Menten or Hill equation using nonlinear regression analysis.

For competitive inhibition experiments, the percent remaining enzyme activity was calculated as the metabolite formation velocity in the presence of an inhibitor relative to that in the absence of the inhibitor. The IC50 for competitive inhibition was estimated by fitting the percent remaining enzyme activity (v) versus the natural logarithm of the initial inhibitor concentration (x) data to the Hill slope equation (eq. 1) using nonlinear regression analysis:

\[
y = \frac{100\%}{1 + 10^\left(\log_{10}\left(\frac{IC_{50}}{x}\right)\right)}.
\] (1)

For time-dependent inhibition experiments, the observed inactivation rate constants (Kobs) were determined from the slopes of the natural logarithm of the percent remaining enzyme activity versus preincubation time plots at varying inhibitor concentrations. The parameters for the maximum rate of inactive enzyme formation (k inactive) and the inhibitor concentration (K) resulting in half of k inactive were estimated by fitting the Kobs versus initial inhibitor concentration (I) plot to eq. 2 using nonlinear regression analysis, assuming that there is negligible change of the inhibitor concentration in the incubation period, and that loss of enzyme is solely due to inactivation by the inhibitor:

\[
K_{obs} = \frac{k_{inactive} \times I}{K + I}.
\] (2)

<table>
<thead>
<tr>
<th>Table 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kinetic parameters for UTL-5g hydrolysis to DCA and ISOX in HLM and by hCE1b and hCE2</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>ISOX formation in HLM</strong></td>
</tr>
<tr>
<td>+ NADPH</td>
</tr>
<tr>
<td>- NADPH</td>
</tr>
<tr>
<td><strong>DCA formation in HLM</strong></td>
</tr>
<tr>
<td>+ NADPH</td>
</tr>
<tr>
<td>- NADPH</td>
</tr>
<tr>
<td><strong>UTL-5g hydrolysis to DCA and ISOX in HLM</strong></td>
</tr>
<tr>
<td>+ NADPH</td>
</tr>
<tr>
<td>- NADPH</td>
</tr>
<tr>
<td><strong>UTL-5g hydrolysis to DCA and ISOX by hCE1b</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>UTL-5g hydrolysis to DCA and ISOX by hCE2</strong></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

*Data are expressed as the estimate (95% confident interval).
*Parameters were estimated by fitting ISOX formation rate versus initial substrate (UTL-5g) concentration data to the Michaelis-Menten equation.
*Parameters were estimated by fitting DCA formation rate versus initial substrate (UTL-5g) concentration data to the Michaelis-Menten equation.
*Parameters were estimated by fitting both ISOX and DCA formation rate versus initial substrate (UTL-5g) concentration pooled data to the Michaelis-Menten equation.
Results

Metabolism of UTL-5g in HLM. In HLM, UTL-5g was rapidly converted to ISOX and DCA. The formation of ISOX or DCA was NADPH-independent (Fig. 2, A and B), suggesting that microsomal P450 enzymes were not involved in the hydrolysis of UTL-5g to ISOX and DCA. The kinetics of UTL-5g hydrolysis to ISOX and DCA in HLM in the presence or absence of NADPH was well described by the Michaelis-Menten equation (Fig. 2, A and B). The kinetic parameters for the formation of ISOX and DCA were not different (Table 3), suggesting that UTL-5g was hydrolyzed into equal molar concentrations of ISOX and DCA. The kinetic parameters in the absence of NADPH were not different from those in the presence of NADPH (Table 3), further indicating that UTL-5g hydrolysis to ISOX and DCA was NADPH-independent. Overall, UTL-5g hydrolysis to ISOX and DCA in HLM was characterized by Michaelis-Menten kinetics, with a $V_{\text{max}}$ of 11.1 nmol/min per mg, $K_m$ of 41.6 μM, and intrinsic clearance of 266.8 μl/min per mg (Table 3).

UTL-5g hydrolysis to ISOX and DCA in HLM in the absence of NADPH was inhibited by NDGA (a known inhibitor of carboxylesterases) with an IC50 of 10.1 μM, quercetin (a known competitive inhibitor of CYP2C8) with an IC50 of 9.8 μM, and ketoconazole (a known competitive inhibitor of CYP3A4/5) with an IC50 of 8.3 μM (Fig. 3).

Enzyme Kinetics of UTL-5g Hydrolysis by Recombinant hCE1b and hCE2. To compare the kinetics of UTL-5g hydrolysis to ISOX and DCA by human carboxylesterases, the recombinant hCE1b (0.04 mg/ml) or hCE2 (0.01 mg/ml) was incubated with varying concentrations of UTL-5g (0.5–100 μM) at 37°C for 10 minutes. The
kinetics of UTL-5g hydrolysis to ISOX and DCA by hCE1b and hCE2 was well described by the Michaelis-Menten equation (Fig. 4). The $K_m$ values for hCE1b and hCE2 were estimated as 26.8 and 12.4 μM, respectively, suggesting hCE2 had ~2-fold higher affinity for UTL-5g than hCE1b. The $V_{max}$ values were 4.3 and 7.0 nmol/min per mg protein for hCE1b and hCE2, respectively. These data suggested that the aniline structure (Fig. 1) was essential to the observed P450 inhibition activity of UTL-5g and its metabolites (DCA and ISOX) was examined in HLM using the known selective P450 probe substrates as the measure of P450 activities (Fig. 5). UTL-5g inhibited CYP2B6 ($IC_{50} \approx 20.6 \mu M$), CYP1A2 ($IC_{50} \approx 30.5 \mu M$), and CYP2C9 (IC$_{50} \approx 45.2 \mu M$), while showing insignificant inhibition for CYP2C8, CYP2C9, CYP2D6, and CYP3A4/5 ($IC_{50} > 50 \mu M$) (Fig. 5; Table 4). Similar to UTL-5g, DCA inhibited CYP1A2 (IC$_{50}$ ~28.9 μM), CYP2B6 (IC$_{50}$ ~31.0 μM), and CYP2C19 (IC$_{50}$ ~83.7 μM), while showing no inhibition of CYP2C8, CYP2C9, CYP2D6, and CYP3A4/5 (IC$_{50}$ $\geq$ 100 μM) (Fig. 5; Table 4). Different from UTL-5g or DCA, ISOX showed insignificant inhibition for all tested microsomal P450 (IC$_{50}$ > 50 μM) (Fig. 5; Table 4). These data suggested that the aniline structure (Fig. 1) was essential to the observed P450 inhibition activity of UTL-5g and DCA. As the positive control, the determined $K_m$ values for known selective P450 competitive inhibitors were consistent with the published data (Table 4).

**Competitive Inhibition of Microsomal P450 by UTL-5g and Its Metabolites.** The P450 inhibition potential of UTL-5g and its metabolites (DCA and ISOX) was examined in HLM using the known selective P450 probe substrates as the measure of P450 activities (Fig. 5). UTL-5g inhibited CYP2B6 ($IC_{50} \approx 20.6 \mu M$), CYP1A2 ($IC_{50} \approx 30.5 \mu M$), and CYP2C9 (IC$_{50} \approx 45.2 \mu M$), while showing insignificant inhibition for CYP2C8, CYP2C9, CYP2D6, and CYP3A4/5 ($IC_{50} > 50 \mu M$) (Fig. 5; Table 4). Similar to UTL-5g, DCA inhibited CYP1A2 (IC$_{50}$ ~28.9 μM), CYP2B6 (IC$_{50}$ ~31.0 μM), and CYP2C19 (IC$_{50}$ ~83.7 μM), while showing no inhibition of CYP2C8, CYP2C9, CYP2D6, and CYP3A4/5 (IC$_{50}$ $\geq$ 100 μM) (Fig. 5; Table 4). Different from UTL-5g or DCA, ISOX showed insignificant inhibition for all tested microsomal P450 (IC$_{50}$ > 50 μM) (Fig. 5; Table 4). These data suggested that the aniline structure (Fig. 1) was essential to the observed P450 inhibition activity of UTL-5g and DCA. As the positive control, the determined $K_m$ values for known selective P450 competitive inhibitors were consistent with the published data (Table 4).

**Time-Dependent Inhibition of Microsomal P450 by UTL-5g and Its Metabolites.** The potential NADPH-, time-, and concentration-dependent inactivation of microsomal P450 by UTL-5g and its metabolites (DCA and ISOX) was examined by preincubating HLM with varying concentrations of UTL-5g, DCA, or ISOX in the presence or absence of NADPH followed by measuring residual individual microsomal P450 activity using the known specific P450 substrate probes. UTL-5g and DCA inhibited microsomal CYP1A2 (using phenacetin conversion to acetaminophen as an indicator of CYP1A2 activity) in a NADPH-, time-, and concentration-dependent manner (Figs. 6 and 7). Different from UTL-5g or DCA, ISOX did not cause time- and concentration-dependent inactivation of any tested microsomal P450 (data not shown). It should be emphasized that UTL-5g or DCA-induced inactivation of CYP1A2 was NADPH-dependent. As shown in Fig. 6, in the absence of NADPH, preincubation of HLM with UTL-5g (50 μM) for 10–40 minutes did not cause an apparent decrease in CYP1A2 activity, whereas when HLM was preincubated with UTL-5g or DCA in the presence of NADPH, CYP1A2 activity declined in a time- and concentration-dependent manner (Fig. 7, A and B). The enzyme inactivation kinetic constants for the time-dependent inhibition of CYP1A2 by UTL-5g and DCA as well as furafylline (a known mechanism-based inactivator of CYP1A2) are presented in Table 5. The determined $K_i$ and $k_{max}$ values for furafylline were consistent with the reported values (Clarke et al., 1994; Teng et al., 2010). UTL-5g and DCA showed similar inactivation kinetic constants. The efficiency ($k_{max}/K_i$) of UTL-5g for inactivating CYP1A2 was ~120-fold lower than that of furafylline (a known mechanism-based inactivator of CYP1A2) (Table 5).

**Discussion**

UTL-5g, acting as a prodrug, is hydrolyzed to a free carboxylic acid, ISOX (Fig. 1), the active metabolite that exerts chemo- and radioprotective activity (Zhang et al., 2014). UTL-5g hydrolysis to equal mols of ISOX and DCA in HLM was NADPH-independent (Fig. 2, A and B; Table 3) and was inhibited by a known esterase inhibitor, NDGA (IC$_{50}$ = 10.1 μM), suggesting that liver microsomal P450 enzymes were not involved, but microsomal esterases played a dominant role in the hydrolysis of UTL-5g. Two major human carboxylesterases, hCE1b and hCE2, effectively catalyzed UTL-5g hydrolysis to ISOX and DCA (Fig. 4; Table 3). When normalized to the unit of enzyme activity, recombinant hCE2 exhibited ~30-fold higher catalytic efficiency ($V_{max}/K_m$) for UTL-5g hydrolysis than hCE1b.

Mammalian carboxylesterases are a family of serine-dependent esterases that catalyze the hydrolysis of a vast array of endogenous and exogenous substrates, including esters, amides, thioesters, and carbamates (Satoh and Hosokawa, 1998). They are located in the cytoplasm and endoplasmic reticulum of virtually all tissues throughout the body, including, but not limited to, the liver, small intestine, kidney, lungs, and brain, with the greatest quantities found in the liver and small intestine (Satoh and Hosokawa, 1998; Laizure et al., 2013). In humans, two major carboxylesterases, hCE1 and hCE2, play an important role in the metabolic activation or inactivation of a number of therapeutic drugs (Laizure et al., 2013). The human liver contains the most abundant expression of hCE1 and smaller quantities of hCE2, whereas the small intestine contains hCE2 with virtually no hCE1.

Both hCE1 and hCE2 contributed to the hydrolysis of UTL-5g to ISOX, but hCE2 was ~30 times more efficient in catalyzing UTL-5g hydrolysis. As hCE2 is predominantly expressed in the small intestine, the hydrolysis of UTL-5g to its active metabolite ISOX would be expected to be more efficient and complete following oral administration than nonoral routes of drug administration (e.g., intravenous, subcutaneous, or intramuscular).
intraperitoneal, subcutaneous, or intramuscular injection) where the drug has no access to the most abundant hCE2 in the small intestine. This speculation was supported by the preliminary pharmacokinetic study in mice showing that after oral administration, UTL-5g was completely hydrolyzed to ISOX and DAC with the parent drug concentration undetectable, whereas after intravenous or intraperitoneal injection, the hydrolysis was relatively slow and incomplete with the parent drug concentration detectable in the system circulation (unpublished data). Based on these data, oral administration is recommended as the route of drug administration for the further development of UTL-5g.

Given the important role of hCE2 and hCE1 in the metabolic activation of UTL-5g, factors influencing carboxylesterase activities may have a significant impact on the pharmacological and therapeutic effects of UTL-5g. There is a growing body of evidence that the activities of carboxylesterases could be modulated by genetic polymorphisms, drug-drug interactions, drug-disease interactions, and other factors (Laizure et al., 2013).

Several genetic variants of potential clinical significance have been identified in the carboxylesterase genes. For example, a commonly occurring nonsynonymous single nucleotide polymorphism (SNP), 428G>A in exon 4 of the hCE1 gene, results in almost complete loss
of hydrolysis activity of the hCE1 enzyme (Zhu et al., 2008). This SNP has been associated with significantly reduced hydrolysis of oseltamivir to its active carboxyl acid metabolite in humans (Tarkiainen et al., 2012). Three functionally deficient SNPs have been identified in the hCE2 gene, including two nonsynonymous SNPs, 100C>T (in exon 2) and 424G>A (in exon 4), and one splice variant (IVS8-2A>G). These functionally deficient SNPs in the hCE1 and hCE2 genes may lead to impaired metabolic activation and thus reduced therapeutic effects of UTL-5g. Further studies are needed to determine the functional and clinical relevance of genetic variants of hCE1 and hCE2 to the pharmacokinetics and pharmacodynamics of UTL-5g.

Besides genetic polymorphisms, carboxylesterase activities can be influenced by a variety of compounds enzymatically and at the transcriptional level (Laizure et al., 2013). It has been reported that the hydrolytic activities of hCE1 or hCE2 are inhibited by ethanol, grapefruit juice, various drugs or compounds (e.g., loperamide, nelfinavir, procainamide, fenofibrate, and NDGA), and herbal natural products, as well as some known P450 inhibitors (Polsky-Fisher et al., 2006; Li et al., 2007; Takahashi et al., 2009; Parker and Laizure, 2010; Li et al., 2007; Takahashi et al., 2009). In addition, carboxylesterases are inducible by a variety of compounds, including some known P450 inducers (e.g., dexamethasone and phenobarbital) (Kaur and Ali, 1983; Ashour et al., 1987; Hosokawa et al., 1988; Zhu et al., 2000; Maruichi et al., 2010). In light of the dominant role of hCE1 and hCE2 in the bioactivation of UTL-5g, it is plausible that inhibition or induction of carboxylesterases caused by drug-drug, drug-food, or drug-disease interactions would alter the therapeutic or adverse effects of UTL-5g.

On the other hand, UTL-5g has the potential to inhibit P450-mediated metabolism through competitive inhibition or time-dependent inhibition of P450 enzymes. In HLM, UTL-5g and one of its hydrolytic metabolites (DCA) competitively inhibited microsomal CYP1A2, CYP2B6, and CYP2C19, with IC50 values <50 μM (Table 4). In addition, UTL-5g and DCA exhibited NADPH, time-, and concentration-dependent inactivation of microsomal CYP1A2, with Kf values <50 μM (Table 5). Different from UTL-5g and DCA, ISOX did not inhibit any of the tested microsomal P450. These data suggest that the aniline (or phenylamine) structure presented in UTL-5g and DCA (Fig. 1) is the essential contributor to the observed competitive inhibition and time-dependent inhibition of microsomal P450. UTL-5g is an amide, whereas DCA contains a primary amine group. The primary amines are known to be the functional groups for potent competitive P450 inhibitors (Polasek and Plopper, 1997).

![Fig. 6. NADPH-dependent inactivation of CYP1A2 in HLM by UTL-5g (A) and DCA (B). HLM was preincubated with UTL-5g (50 μM) for 10–40 minutes in the absence or presence of NADPH-regenerating system followed by measurement of residual CYP1A2 activity (i.e., phenacetin conversion to acetaminophen) in the secondary incubation. Each data point represents the mean of duplicate samples. The solid line represents the fitting of observed data using linear regression.](image-url)
Miners, 2008). These functional amine groups are also a common feature for time-dependent inactivators (Polasek and Miners, 2008). Given the fact that UTL-5g is rapidly hydrolyzed to DCA in HLM, the observed UTL-5g–induced competitive inhibition and time-dependent inhibition of P450 could be attributable to the effect of DCA. This possibility was supported by the observation that UTL-5g and DCA exhibited similar IC₅₀ values for competitive inhibition CYP1A2, CYP2B6, and CYP2C19 (Fig. 5; Table 4) as well as a similar kₘᵢₙ/kₐᵢ value for time-dependent inhibition of CYP1A2 (Fig. 7; Table 5). The proposed pathways of UTL-5g metabolism and potential for competitive and time-dependent inhibition of P450 are summarized in Fig. 1. The exact biochemical mechanisms underlying time-dependent inhibition of CYP1A2 by UTL-5g and DCA are yet to be determined.

![Fig. 7. (A, C, and E) Time- and concentration-dependent inactivation of CYP1A2 in HLM by UTL-5g, DCA, and furafylline (a known mechanism-based inactivator of CYP1A2). Microsomal CYP1A2 activity was assessed by measuring the conversion of phenacetin to acetaminophen. Each data point represents the mean of duplicate samples. The solid line represents the fitting of the observed natural logarithm of the percent remaining CYP1A2 activity versus preincubation time data using linear regression. (B, D, and F) Inactivation rate constant (kₘᵢₙ) versus inhibitor concentration plots for UTL-5g, DCA, and furafylline. Each data point represents the observed kₘᵢₙ that is determined from the slope of the natural logarithm of the percent remaining enzyme activity versus preincubation time plots at a particular inhibitor concentration. The curve represents the fitting of observed kₘᵢₙ versus inhibitor concentration data to eq. 2.](image)

<table>
<thead>
<tr>
<th>Inactivator</th>
<th>kᵢ (µM)</th>
<th>kₘᵢₙ (µM⁻¹ min⁻¹)</th>
<th>kₘᵢₙ/kᵢ (µM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UTL-5g</td>
<td>22.2</td>
<td>0.015</td>
<td>0.68</td>
</tr>
<tr>
<td>DCA</td>
<td>45.3</td>
<td>0.023</td>
<td>0.51</td>
</tr>
<tr>
<td>ISOX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Furafylline (a known CYP1A2 mechanism-based inactivator)⁻</td>
<td>0.4</td>
<td>0.033</td>
<td>82.5</td>
</tr>
</tbody>
</table>

⁻Kinetic constants not estimated, no inactivation detected.
⁻Furafylline (a known CYP1A2 mechanism-based inactivator) was used as the positive control for mechanism-based inactivation of CYP1A2 (using phenacetin conversion to acetaminophen as an indicator of CYP1A2 activity).
It should be noted that UTL-5g and DCA are both competitive inhibitors (with IC₅₀ values of ~30 μM) and time-dependent inactivators (with Kᵣ values of 22 and 45 μM, respectively) for CYP1A2. Time-dependent inhibition is distinguished from competitive (or reversible) inhibition in that enzymatic activity can only be restored through de novo protein synthesis, and thus inhibition persists in vivo even after the elimination of the inactivator. The inactivation efficiency (kₐ/diss/Kᵣ) of UTL-5g and DCA is considerably lower than that of furafylline. A known mechanism-based inactivator of CYP1A2 (Table 5). Although in vivo data are not available at present, the extensive first-pass metabolism of UTL-5g to DCA in the intestine and liver after oral administration, DCA concentrations in the liver would be expected to be higher than in vitro IC₅₀ or Kᵣ values for competitive inhibition or time-dependent inhibition of CYP1A2. Therefore, the oral administration of UTL-5g has the potential to cause a clinically relevant increase in the systemic exposure of a coadministered drug inactivator, from porcine as follows: P450-mediated metabolism: have we overlooked their importance? Pharmacotherapy 33:210–222.


Authorship Contributions

Performed experiments: Wu, Dubaisi.

Conducted experiments: Li, Shaw, Wu.

Contributed new reagents or analytic tools: Shaw, Valeriote, Li, Wu.

Wrote or contributed to the writing of the manuscript: Li, Wu, Dubaisi.

References


In vitro metabolism and drug-drug interaction potential of UTL-5g, a novel chemo- and radio-protective agent

Jianmei Wu, Jiajiu Shaw, Sarah Dubaisi, Frederick Valeriote, and Jing Li

Karmanos Cancer Institute, Department of Oncology, Wayne State University School of Medicine, Detroit, MI 48201 (JW, JL)

21st Century Therapeutics, Inc., Ferndale, MI 48220 (JS)

Department of Pharmacology, Wayne State University School of Medicine, Detroit, MI 48201 (SD)

Department of Internal Medicine, Henry Ford Health System, Detroit, MI 48202 (FV)

Supplementary Figure 1 Total ion chromatogram of the P450 probe cocktail metabolites and internal standards in PBS/acetonitrile (1:4, v/v) containing acetaminophen (4 µM), OH-bupropion (4 µM), 1’-OH midazolam (4 µM), 5-OH-rosiglitazone (0.4 µM), dextrophan (4 µM), S-4-OH-mephenytoin (20 µM), 4’-OH-diclofenac (20 µM). Chromatographic separation was performed on Nova-Pak C18 column (4 µm, 3.9 × 150 mm) at 30°C, running with a gradient mobile phase consisting of 10mM ammonium formate (pH 7.5) (A) and methanol (B) at a flow rate of 0.4 mL/min, with mobile phase gradient B% (min) programmed as 55 (0) → 100 (10) → 55 (10.1) → 100 (16). MS transitions are shown in Table 2.
**Supplementary Figure 2** Calibration curves for P450 cocktail probe metabolites. Calibration curves were prepared by spiking known concentrations of metabolite reference standards in PBS at the concentration ranges of 2 – 2000 nM for dextrophan and 5-OH-rosiglitazone, 5 – 2000 nM for OH-bupropion and 1’-OH-midazolam, 20 – 2000 nM for acetaminophen, 50 – 2000 nM for S-4-OH-mephenytoin, and 100 – 2000 nM for 4’-OH-diclofenac. The calibrator PBS sample was added with 4-fold acetonitrile, vortex-mixed, and the mixture was subjected to LC-MS/MS analysis. Internal standard method with isotope-labeled internal standards was used for quantitation of acetaminophen, OH-bupropion, 1’-OH-midazolam, and 5-OH-rosiglitazone; external standard method was used for quantitation of dextrophan, S-4-OH-mephenytoin, and 4’-OH-diclofenac, for which isotope-labeled internal standards were not available.
Supplementary Figure 3 Metabolic kinetics of P450 cocktail probes. Kinetic parameters ($V_{\text{max}}$ and $K_{\text{m}}$) were estimated by fitting metabolite formation rate versus initial substrate concentration curve to the Michaelis-Menten equation using non-linear regression. Microsomal P450 activity was assessed by measuring the conversion of a known specific probe substrate to metabolite: phenacetin $\rightarrow$ acetaminophen for CYP1A2, bupropion $\rightarrow$ OH-bupropion for CYP2B6, rosiglitazone $\rightarrow$ 5-OH-rosiglitazone for CYP2C8, diclofenac $\rightarrow$ 4'-OH-diclofenac for CYP2C9, S-mephenytoin $\rightarrow$ S-4'-OH-mephenytoin for CYP2C19, dextromethorphan $\rightarrow$ Dextrorphan for CYP2D6, midazolam $\rightarrow$ 1'-OH-midazolam for CYP3A4/5.