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

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ABSTRACT

N-(2,4-dichlorophenyl)-5-methyl-1,2-oxazole-3-carboxamide (UTL-5g), a potential chemo- and radioprotective agent, acts as a prodrug 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 ($V_{\max}$) of 11.1 nmol/min per mg and substrate affinity (K_m) of 41.6 μ M. Both hCE1b and hCE2 effectively catalyzed UTL-5g hydrolysis, but

hCE2 exhibited ~30-fold higher catalytic efficiency ($V_{\max}/K_m$) than hCE1b. UTL-5g and DCA competitively inhibited microsomal CYP1A2, CYP2B6, and CYP2C19 (IC_{50} values <50 μ M), and exhibited time-dependent inhibition of microsomal CYP1A2 with the inactivation efficiency (k_{inact}/K_i) of 0.68 and 0.51 $\text{minute}^{-1}\cdot\text{mM}^{-1}$, 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 free 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 higher 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 hematology 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).

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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.

Fig. 1. Proposed pathways of UTL-5g metabolism and potential for competitive inhibition and time-dependent inhibition of microsomal P450. *The observed UTL-5g-induced competitive inhibition and time-dependent inhibition of P450 could be attributable to DCA given the rapid hydrolysis of UTL-5g to DCA in HLM.

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 and use of UTL-5g as a potential chemo- and radioprotective agent in humans.

In this study, we characterized the metabolism of UTL-5g in pooled human liver microsomes (HLM), and determined the kinetics of UTL-5g hydrolysis by two recombinant human carboxylesterase enzymes, hCE1b and hCE2. In addition, we evaluated potential interactions of UTL-5g and its metabolites (ISOX and DCA) with microsomal cytochrome P450 (P450) enzymes.

Materials and Methods

Chemicals and Reagents. UTL-5g (lot#1182-MEM-3D, purity >99%) was synthesized at Kalexsyn Medicinal Chemistry (Kalamazoo, MI). ISOX and DCA were purchased from Sigma-Aldrich (Kalamazoo, MI). Phenacetin,

acetaminophen, diclofenac, rosiglitazone, furafylline, ketoconazole, sulfaphenazole, benzylnirvanol, quinidine, and quercetin were purchased from Sigma-Aldrich (St. Louis, MO); hydroxy bupropion, bupropion, *S*-mephenytoin, diclofenac, and hydroxyl bupropion-*d*₆ were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX). *S*-4-hydroxy mephenytoin, dextropran, dextromethorphan, 5-hydroxy rosiglitazone, midazolam, 1'-hydroxy midazolam, and 5-hydroxy rosiglitazone-*d*₄ were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). 4'-Hydroxy-diclofenac was obtained from Cayman Chemical (Ann Arbor, MI). Acetaminophen-*d*₄ was purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada). 1'-Hydroxy midazolam-*d*₄ was obtained from Cerilliant (Round Rock, TX). BD UltraPool HLM 150 (150-donor human liver microsome pools with equal gender mix), recombinant hCE1b and hCE2, CYP1A2 Supersomes, as well as NADPH regenerating system solution A and B were obtained from Corning Inc. (Woburn, MA).

Metabolism of UTL-5g in HLM and by Recombinant hCE1b and hCE2.

Preliminary experiments were conducted to determine the optimal conditions in which linear product formation occurs with respect to HLM protein concentration and incubation time. Subsequent experiments were conducted using the linear product formation conditions (0.05 mg/ml HLM, 10 minutes). The kinetics of UTL-5g hydrolysis to ISOX and DCA in HLM was examined by incubating varying concentrations of UTL-5g (0.5–100 μ M) with HLM (0.05 mg/ml) in the presence or absence of NADPH at 37°C in a shaking water bath for 10 minutes. In the presence of NADPH, the reaction mixture (total volume 0.2 ml) contained UTL-5g (0.5–100 μ M), HLM (0.05 mg/ml), and NADPH-regenerating system (including 1.3 mM NADP⁺, 3.3 mM glucose-6-phosphate, 0.4 units/ml glucose-6-phosphate dehydrogenase, and 3.3 mM magnesium chloride) in 100 mM potassium phosphate-buffered solution (PBS) (pH 7.4). In the absence of NADPH, NADPH-regenerating system was replaced by PBS.

The effects of the known P450 inhibitors on UTL-5g hydrolysis were examined by incubating UTL-5g (10 μ M) with varying concentrations (0.1–100 μ M) of the inhibitor for CYP1A2 (furafylline), CYP2C8 (quercetin), CYP2C9 (sulfaphenazole), CYP2C19 (benzylnirvanol), CYP2D6 (quinidine), or CYP3A4/5 (ketoconazole) in the absence of NADPH at 37°C in a shaking water bath for 10 minutes. In addition, the effect of a specific carboxylesterase inhibitor, nordihydroguaiaretic acid (NDGA) (Takahashi et al., 2009), on

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

	UTL-5g	DCA	ISOX	Zileuton (Internal Standard)
MS mode	Positive	Positive	Positive	Positive
<i>m/z</i> transition	271.17 > 109.96	161.92 > 125.95	128.05 > 109.96	237.08 > 161.03
Capillary voltage (Kv)	3	3	3	3
Cone voltage (V)	20	35	15	16
Collision energy (Ev)	16	18	10	12
Desolvation temperature (°C)	350	350	350	350
Source temperature (°C)	120	120	120	120
Retention time (min)	16.86	14.63	11.85	13.53
Mobile phase gradient program: %B (min) ^a	10 (0) → 95 (3.5) → 95 (19) → 10 (19.5) → 10 (24)			

^aMobile phase consisted of water (containing 0.1% formic acid) (A) and methanol (containing 0.1% formic acid) (B).

TABLE 2

LC-MS/MS parameters for quantitation of the known microsomal P450 probe metabolites

Microsomal P450 activity was assessed by the conversion of a known specific probe substrate to metabolite: phenacetin → acetaminophen for CYP1A2, bupropion → OH-bupropion for CYP2B6, rosiglitazone → 5-OH-rosiglitazone for CYP2C8, diclofenac → 4'-OH-diclofenac for CYP2C9, S-mephenytoin → S-4-hydroxy mephenytoin for CYP2C19, dextromethorphan → dextrorphan for CYP2D6, midazolam → 1'-OH-midazolam for CYP3A4/5.

	Acetaminophen (CYP1A2)	OH-bupropion (CYP2B6)	5-OH-rosiglitazone (CYP2C8)	4'-OH-diclofenac (CYP2C9)	S-4-OH-mephenytoin (CYP2C19)	Dextrorphan (CYP2D6)	1'-OH-midazolam (CYP3A4/5)
Internal standard	Acetaminophen- <i>d</i> ₄	OH-bupropion- <i>d</i> ₆	5-OH-rosiglitazone- <i>d</i> ₄	N/A ^a	N/A ^a	N/A ^a	1'-OH-midazolam- <i>d</i> ₄
MS mode	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Analyte <i>m/z</i> transition	152.04 > 109.93	256.22 > 238.23	374.19 > 151.07	312.05 > 231.15	235.24 > 150.09	258.24 > 157.08	341.93 > 324.14
Internal standard <i>m/z</i> transition	156.1 > 114.0	262.25 > 244.27	378.24 > 155.06	N/A ^a	N/A ^a	N/A ^a	346.17 > 328.13
Capillary voltage (Kv)	3	3	3	3	3	3	3
Cone voltage (V)	25	16	38	22	30	30	34
Collision energy (Ev)	15	12	24	18	18	35	20
Desolvation temperature (°C)	350	350	350	350	350	350	350
Source temperature (°C)	120	120	120	120	120	120	120
Standard curve range (nM)	20 ~2000	5 ~2000	2~2000	100 ~2000	50 ~2000	2 ~2000	5 ~2000
Retention time (min)	2.9	8.1	6.8	4.2	3.7	6.9	9.3
Mobile phase gradient: %B (min) ^b			55 (0) → 100 (10) → 55 (10.1) → 100 (16)				

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

^bMobile phase consisted of 10 mM ammonium formate (pH 7.5) (A) and methanol (B).

UTL-5g hydrolysis was examined by incubating UTL-5g (40 μ M) with NDGA (1–100 μ M) 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 μ M) 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 microsome or enzyme reaction, the reaction (200 μ l) was quenched with 800 μ l of ice-cold acetonitrile containing 0.1% formic acid and zileuton (1 μ M) 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 μ m, 3.9 $\times$ 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 μ M. 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 (dextrorphan), 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 μ M), bupropion (5–320 μ M), rosiglitazone (1.25–80 μ M), diclofenac (0.625–40 μ M), S-mephenytoin

(5–320 μ M), dextromethorphan (2.5–160 μ M), and midazolam (0.625–40 μ M) 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 units/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 μ l) was quenched with 800 μ l 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.

The P450 probe substrates and their metabolites in the supernatant were simultaneously determined using a Waters 2695 high-performance liquid chromatography system coupled with a Waters Quattro Micro triple quadrupole mass spectrometer (Waters). Chromatographic separation was performed on a Nova-Pak C18 column (4 μ m, 3.9 $\times$ 150 mm; Waters) at 4°C, running at a gradient mobile phase consisting of 10 mM ammonium formate (pH 7.5) (A) and methanol (B) at a flow rate of 0.4 ml/min. Table 2 shows the analytical parameters for the analytes. The total ion chromatogram for P450 probe metabolites is shown in Supplemental Fig. 1. Representative calibration curves for individual metabolites are shown in Supplemental Fig. 2. Intra- and interday precisions and accuracies for quality control samples were <15%.

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 incubating varying concentrations of UTL-5g, DCA, or ISOX with the P450 probe cocktail at the probe concentrations equal to their respective K_m 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 μ M), phenacetin (35 μ M), bupropion (20 μ M), rosiglitazone (5 μ M), diclofenac (3 μ M), S-mephenytoin (55 μ M), dextromethorphan (3 μ M), midazolam (2 μ M), 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 μ l) was quenched with 800 μ l 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.

Fig. 2. UTL-5g hydrolysis to ISOX (A) and DCA (B) in HLM (0.05 mg/ml) in the absence and presence of NADPH. Each data point represents the mean of duplicate samples. The curve represents the fitting of the observed data to the Michaelis-Menten equation.

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 K_m 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 14,000g 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 ($C_{m,t}/t$ per P450 concentration), where $C_{m,t}$ is the metabolite concentration at the end of incubation and t is the incubation time. The velocity (v) versus initial substrate concentration ($C_{s,0}$) plots as well as corresponding Eadie-Hofstee ($v/C_{s,0}$ versus $C_{s,0}$) and Lineweaver-Burk ($1/v$ versus $1/C_{s,0}$) plots were constructed. The metabolic kinetic parameters for maximum rate of reaction (V_{max}) and substrate concentration at which 50% of the maximum reaction rate is achieved (K_m) were estimated by fitting the v versus $C_{s,0}$ 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 IC_{50} for competitive inhibition was estimated by fitting the percent remaining enzyme activity (y) versus the natural logarithm of the initial inhibitor concentration (x) data to the Hill slope equation (eq. 1) using nonlinear regression analysis:

$$y = \frac{100\%}{1 + 10^{n(\log 10x - \log 10IC_{50})}} \quad (1)$$

For time-dependent inhibition experiments, the observed inactivation rate constants (K_{obs}) 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_{inact}) and the inhibitor concentration (K_I) resulting in half of k_{inact} were estimated by fitting the K_{obs} 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:

$$K_{obs} = \frac{k_{inact} \times I}{K_I + I} \quad (2)$$

TABLE 3
Kinetic parameters for UTL-5g hydrolysis to DCA and ISOX in HLM and by hCE1b and hCE2

	V_{max}^a nmol/min per mg	K_m^a μM
ISOX formation in HLM ^b		
+ NADPH	11.6 (10.0–13.2)	43.2 (30.0–56.5)
– NADPH	11.4 (10.0–12.8)	51.2 (38.2–64.1)
DCA formation in HLM ^c		
+ NADPH	11.1 (9.6–12.5)	34.6 (23.6–45.5)
– NADPH	10.4 (8.9–11.9)	39.4 (26.2–52.5)
UTL-5g hydrolysis to DCA and ISOX in HLM ^d	11.1 (10.3–11.9)	41.6 (34.7–48.4)
UTL-5g hydrolysis to DCA and ISOX by hCE1b ^d	4.3 (3.9–4.7)	26.8 (20.4–33.3)
UTL-5g hydrolysis to DCA and ISOX by hCE2 ^d	7.0 (6.3–7.8)	12.4 (8.3–16.6)

^aData are expressed as the estimate (95% confident interval).

^bParameters were estimated by fitting ISOX formation rate versus initial substrate (UTL-5g) concentration data to the Michaelis-Menten equation.

^cParameters were estimated by fitting DCA formation rate versus initial substrate (UTL-5g) concentration data to the Michaelis-Menten equation.

^dParameters were estimated by fitting both ISOX and DCA formation rate versus initial substrate (UTL-5g) concentration pooled data to the Michaelis-Menten equation.

Fig. 3. Inhibitory effects of the known P450 inhibitors, including furaflavone (CYP1A2) (A), quercetin (CYP2C8) (B), sulfaphenazole (CYP2C9) (C), benznirvanol (CYP2C19) (D), quinidine (CYP2D6) (E), and ketoconazole (CYP3A4/5) (F), as well as a known carboxylesterase inhibitor (NDGA) (G), on UTL-5g hydrolysis to ISOX and DCA in HLM. Each data point represents the mean of duplicate samples. The curve represents the fitting of the observed ISOX or DCA formation rate (% control) (y) versus the natural logarithm of the initial inhibitor concentration (x) data to the Hill slope equation (eq. 1).

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 $V_{\max}$ of 11.1 nmol/min per mg, K_m of 41.6 μM , and intrinsic clearance of 266.8 $\mu\text{L}/\text{min}$ per mg (Table 3).

UTL-5g hydrolysis to ISOX and DCA in the absence of NADPH was inhibited by NDGA (a known inhibitor of carboxylesterases) with an IC_{50} of 10.1 μM , quercetin (a known competitive inhibitor of CYP2C8) with an IC_{50} of 9.8 μM , and ketoconazole (a known competitive inhibitor of CYP3A4/5) with an IC_{50} 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. The V_{max} values were then normalized to the unit of enzyme activity based on the manufacturer-determined enzyme activity using 4-nitrophenyl acetate assay, i.e., 270.8 and 31.2 units/mg protein for recombinant hCE1b and hCE2, respectively. Hence, the normalized V_{max} values of UTL-5g hydrolysis by hCE1b and hCE2 were calculated as 15.9 and 224.0 pmol/min per unit enzyme, respectively, suggesting that hCE2 had ~ 14 -fold higher catalytic capability for UTL-5g hydrolysis compared with hCE1b. Overall, the catalytic efficiency (V_{max}/K_m) of hCE2 (18.1 $\mu\text{l}/\text{min}$ per unit enzyme) was ~ 30 -fold higher than that of hCE1b (0.6 $\mu\text{l}/\text{min}$ per unit enzyme).

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 ($\text{IC}_{50} \sim 20.6 \mu\text{M}$), CYP1A2 ($\text{IC}_{50} \sim 30.5 \mu\text{M}$), and CYP2C19 ($\text{IC}_{50} \sim 45.2 \mu\text{M}$), while showing insignificant inhibition for CYP2C8, CYP2C9, CYP2D6, and CYP3A4/5 ($\text{IC}_{50} > 50 \mu\text{M}$) (Fig. 5; Table 4). Similar to UTL-5g, DCA inhibited CYP1A2 ($\text{IC}_{50} \sim 28.9 \mu\text{M}$), CYP2B6 ($\text{IC}_{50} \sim 31.0 \mu\text{M}$), and CYP2C19 ($\text{IC}_{50} \sim 83.7 \mu\text{M}$), while showing no inhibition of CYP2C8, CYP2C9, CYP2D6, and CYP3A4/5 ($\text{IC}_{50} \geq 100 \mu\text{M}$) (Fig. 5; Table 4). Different from UTL-5g or DCA, ISOX showed insignificant inhibition for all tested microsomal P450 ($\text{IC}_{50} > 50 \mu\text{M}$) (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 IC_{50} 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_{inact} 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_{inact}/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 molar 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 ($\text{IC}_{50} = 10.1 \mu\text{M}$), 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,

Fig. 4. UTL-5g hydrolysis to ISOX and DCA in hCE1b (A) and hCE2 (B) in the absence of NADPH. Each data point represents a single measurement. The curve represents the fitting of both ISOX and DCA formation rate versus initial UTL-5g concentration pooled data from two independent experiments to the Michaelis-Menten equation.

Fig. 5. Competitive inhibition of microsomal P450 activity by UTL-5g and its metabolites (DCA and ISOX). Microsomal P450 activity was assessed by the conversion of a known specific probe substrate to metabolite: phenacetin → acetaminophen for CYP1A2 (A), bupropion → OH-bupropion for CYP2B6 (B), rosiglitazone → 5-OH-rosiglitazone for CYP2C8 (C), diclofenac → 4'-OH-diclofenac for CYP2C9 (D), *S*-mephenytoin → 5-OH-mephenytoin for CYP2C19 (E), dextromethorphan → dextrorphan for CYP2D6 (F), and midazolam → 1'-OH-midazolam for CYP3A4/5 (G). Percent remaining activity is the formation rate of a probe metabolite in the presence of the inhibitor (i.e., UTL-5g, DCA, or ISOX) relative to that in the absence of the inhibitor. Each data point represents the mean of duplicate samples. The curve represents the fitting of the percent remaining enzyme activity (y) versus natural logarithm of the initial inhibitor concentration (x) data to the Hill slope equation (eq. 1).

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

TABLE 4

Competitive inhibition of microsomal P450 activity by UTL-5g, its metabolites (DCA and ISOX), and known P450 inhibitors in human liver microsomes

Microsomal P450 activity was assessed by measuring the conversion of a known specific probe substrate to metabolite: phenacetin → acetaminophen for CYP1A2, bupropion → OH-bupropion for CYP2B6, rosiglitazone → 5-OH-rosiglitazone for CYP2C8, diclofenac → 4'-OH-diclofenac for CYP2C9, S-mephenytoin → S-4-OH-mephenytoin for CYP2C19, dextromethorphan → dextrorphan for CYP2D6, midazolam → 1'-OH-midazolam for CYP3A4/5.

	IC ₅₀ (95% Confidence Interval) ^a						
	CYP1A2	CYP2B6	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP3A4/5
UTL-5g	30.5 (25.9–36.0)	20.6 (17.1–24.8)	61.2 (55.1–68.0)	>100	45.2 (29.1–70.2)	81.8 (65.8–102)	>100
DCA	28.9 (17.1–24.8)	31.05 (26.1–36.9)	99.4 (73.0–135)	>100	83.7 (28.8–242)	>100	>100
ISOX	>100	61.52 (52.2–72.5)	>100	>100	>100	>100	>100
Furafylline	1.5 (1.2–2.0)						
Ticlopidine		0.16 (0.07–0.36)					
Quercetin			12.3 (10.8–14.1)				
Sulfaphenazole				0.42 (0.32–0.56)			
Benzylrinivorol					2.1 (0.8–5.5)		
Quinidine						0.10 (0.06–0.16)	
Ketoconazole							0.08 (0.07–0.10)

^aIC₅₀ (95% confidence interval) was estimated by fitting the percent remaining enzyme activity (y) versus natural logarithm of the initial inhibitor concentration (x) pooled data (from duplicate measurements) to the Hill slope equation (eq. 1) using nonlinear regression analysis.

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; Laizure et al., 2013). In the present study, UTL-5g hydrolysis to ISOX and DCA in HLM in the absence of NADPH was inhibited by the known carboxylesterase inhibitor NDGA (IC₅₀ = 10.1 μM), CYP3A4/5 inhibitor ketoconazole (IC₅₀ = 8.3 μM), and CYP2C8 inhibitor quercetin (IC₅₀ = 9.8 μM) (Fig. 3). It should be noted that the inhibition of UTL-5g hydrolysis by ketoconazole or quercetin was NADPH-independent, indicating that the observed drug-drug interaction was not attributable to

competitive inhibition of microsomal P450 (i.e., CYP3A4/5 or CYP2C8), but was likely caused by inhibition of microsomal esterases. Indeed, it has been reported that ketoconazole, quercetin, and some other P450 inhibitors can inhibit human liver microsomal esterase activity (Polsky-Fisher et al., 2006; 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 *K_i* 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

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.

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 (K_{obs}) versus inhibitor concentration plots for UTL-5g, DCA, and furafylline. Each data point represents the observed K_{obs} 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 K_{obs} versus inhibitor concentration data to eq. 2.

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 IC_{50} values for competitive inhibition

CYP1A2, CYP2B6, and CYP2C19 (Fig. 5; Table 4) as well as a similar k_{inact}/K_I 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.

TABLE 5
Inactivation kinetic constants of UTL-5g and its metabolites (DCA and ISOX) for time-dependent inhibition of CYP1A2 in human liver microsomes

Inactivator	K_I	k_{inact}	k_{inact}/K_I
	μM	min^{-1}	$min^{-1} \cdot mM^{-1}$
UTL-5g	22.2	0.015	0.68
DCA	45.3	0.023	0.51
ISOX	— ^a	— ^a	— ^a
Furafylline (a known CYP1A2 mechanism-based inactivator) ^b	0.4	0.033	82.5

^aKinetic constants not estimated, no inactivation detected.
^bFurafylline (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 IC_{50} values of $\sim 30 \mu M$) and time-dependent inactivators (with K_I values of 22 and $45 \mu M$, 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 (k_{inact}/K_I) of UTL-5g and DCA is considerably lower than that of furafylline, a strong mechanism-based inactivator of CYP1A2 (Table 5). Although in vivo data are not available at present, given 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 IC_{50} or K_I 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 for which CYP1A2 metabolism is a major elimination pathway. In fact, zileuton, a known mechanism-based inactivator of CYP1A2 with a similar inactivation efficiency (k_{inact}/K_I) as that of UTL-5g or DCA (Lu et al., 2003), elicits clinical drug interactions with CYP1A2 substrate drugs (e.g., theophylline, *R*-warfarin, and antipyrine) (Awni et al., 1995; Granneman et al., 1995; St Peter et al., 1995). Although clinical studies are eventually required to confirm clinically significant drug interactions, caution is needed on potential drug interactions involving reversible and irreversible inhibition of CYP1A2 in the future clinical development of UTL-5g.

In conclusion, human carboxylesterases hCE1 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. On the other hand, UTL-5g has the potential to inhibit P450-mediated metabolism through competitive inhibition or time-dependent inhibition. Caution is particularly needed on potential drug interactions involving competitive inhibition or time-dependent inhibition of CYP1A2 in the future clinical development of UTL-5g.

Authorship Contributions

Participated in research design: Li, Shaw, Wu.

Conducted experiments: Wu, Dubaisi.

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

Performed data analysis: Li, Dubaisi.

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

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