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Using a simple HPLC approach to identify the enzymatic products of UTL-5g, a small molecule TNF- α inhibitor, from porcine esterase and from rabbit esterase

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Abstract

UTL-5g is a novel small-molecule chemoprotector that lowers hepatotoxicity, nephrotoxicity, and myelotoxicity induced by cisplatin through TNF- inhibition among other factors. As a prelude to investigating the metabolites of UTL-5g, we set out to identify the enzymatic products of UTL-5g under the treatment of both porcine liver esterase (PLE) and rabbit liver esterase (RLE). First, a number of mixtures made by UTL-5g and PLE were incubated at 25 °C. At predetermined time points, individual samples were quenched by acetonitrile, vortexed, and centrifuged. The supernatants were then analyzed by reversed-phase HPLC (using a C18 column). The retention times and UV/Vis spectra of individual peaks were compared to those of UTL-5g and its two postulated enzymatic products; thus the enzymatic products of UTL-5g were tentatively identified. Secondly, a different HPLC method (providing different retentions times) was used to cross-check and to confirm the identities of the two enzymatic products. Based on the observations, it was concluded that under the treatment of PLE, the major enzymatic products of UTL-5g were 5-methylisoxazole-3-carboxylic acid (ISOX) and 2,4-dichloroaniline (DCA). Treatment of UTL-5g by RLE also provided the same enzymatic products of UTL-5g from esterase. These results indicate that the peptide bond in UTL-5g was cleaved by PLE/RLE. Michaelis–Menten kinetics showed that the K_m values of UTL-5g were 2.07 mM with PLE and 0.37 mM with RLE indicating that UTL-5g had a higher affinity with RLE. In summary, by a simple HPLC approach, we have concluded that the peptide bond in UTL-5g was cleaved by esterase from either porcine liver or rabbit liver *in vitro* and afforded DCA (at a mole ratio of 1:1) and ISOX. However, further studies are needed in order to determine whether UTL-5g is metabolized by microsomal enzymes to produce ISOX and DCA.

Keywords

Esterase; enzymatic products; HPLC approach; peptide bond; metabolite; Michaelis–Menten kinetics

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1. INTRODUCTION

UTL-5g (Fig.1) is a novel small-molecule tumor necrosis factor- α (TNF- α) inhibitor having several beneficial biological properties. Briefly UTL-5g is chemoprotective against cisplatin-induced hepatotoxicity, nephrotoxicity, and myelotoxicity as indicated by lowering elevated levels of aspartate transaminase (AST), alanine transaminase (ALT), creatinine, blood urea nitrogen (BUN), and TNF- α as well as by increasing the reduced platelet count in mice [1]. UTL-5g is also radioprotective against radiation-induced acute liver toxicity as indicated by lowering elevated levels of AST, ALT, and TNF- α [2]. In developing a therapeutic agent, it is important to identify its metabolites and to investigate the metabolic activities. As a prelude of our effort in investigating the potential metabolites of UTL-5g, we set out to conduct this *in vitro* study to identify the enzymatic products of UTL-5g under the treatment of both porcine esterase and rabbit esterase individually. Further, a simple HPLC approach was used for the identification of the enzymatic products of UTL-5g.

Structurally, UTL-5g is based on a molecular scaffold, 5-methylisoxazole-3-carboxamide, which is similar to that of leflunomide, 5-methylisoxazole-4-carboxamide; leflunomide (Fig. 1) (sold as Arava® by Sonafi-Aventis) is a disease-modifying antirheumatic drug (DMARD) approved for the treatment of rheumatoid arthritis (RA) [3–5]. When leflunomide is metabolized, its isoxazole ring is cleaved open to produce its active metabolite, teriflunomide, also referred to as A77 1726 [6, 7] (Fig. 1). As reported by Kalgutkar et al., an unsubstituted C3 on the isoxazole is essential for the opening of isoxazole ring [7], which is the case for leflunomide, wherein the isoxazole ring was opened by cleavage of the N-O bond upon metabolism. Since UTL-5g has a substituted C3, we hypothesize that the isoxazole ring should not be metabolically opened. In this work, we set out to use a simple HPLC approach to identify the enzymatic products of UTL-5g and show that the isoxazole ring of UTL-5g is not cleaved opened by esterase.

Esterase functions as an enzyme that hydrolyzes an ester into an acid and an alcohol; it is found in liver, blood, intestine, and other tissues and is of clinical significance in human [8, 9]. Although most *in vitro* metabolic investigations are conducted with microsome treatment [10–13], esterase in plasma and red blood cells (RBC) is reported to be active in drug metabolism in some cases [9]. Therefore, it is conceivable that treatment of esterase may provide some valuable information pertaining to the metabolism of UTL-5g.

In addition to the normal function of hydrolyzing an ester, PLE has been commonly used in research including the asymmetric synthesis in organic chemistry [14, 15]. RLE has been used to investigate the toxic effect of carbamate insecticides [16] and the effect of MK-733 (simvastatin) on intestinal acylcoenzyme A [17]. Furthermore, both esterases are commercially available. Therefore, PLE and RLE were selected for this preliminary investigation on the potential metabolites of UTL-5g.

2. MATERIALS AND METHODS

2.1. Materials

UTL-5g (Lot#1182-MEM-3D, Purity > 99%) was synthesized at Kalexsyn Medicinal Chemistry, Kalamazoo, Michigan. Porcine liver esterase (PLE), rabbit liver esterase (RLE), 5-isoxazole-3-carboxylic acid (ISOX), and 2,4-dichloroaniline (DCA) were purchased from Sigma-Aldrich. HPLC solvents were purchased from Burdick and Jackson. Hank's balanced salt solution was purchased from Cellgro. All other chemicals and solvents were purchased from Sigma-Aldrich unless otherwise specified.

2.2. Methods

UTL-5g was first treated with PLE and the major enzymatic products under the treatment of PLE were investigated by HPLC employing a C18 column. Secondly, a different HPLC method (using a C8 column and different mobile phase parameters) was used to cross-check and confirm the enzymatic products of UTL-5g from PLE. For the enzymatic products of UTL-5g under RLE treatment, the same procedure was used. In addition, Michaelis–Menten kinetic analysis was conducted to derive and compare the maximum reaction rate ($V_{\max}$) and K_m (substrate concentration at which the reaction rate is half of $V_{\max}$) for UTL-5g with these two esterases.

Briefly, 5 μ L of UTL-5g in acetonitrile (2.71 mg/mL) was added into a number of microtubes, each containing 200 μ L of porcine esterase in Hank's Balanced Salt solution without calcium and magnesium (pH 7.25, final concentration 21 unit/mL) and incubated at 25 °C. At predetermined time points, individual samples were quenched by adding 800 μ L of acetonitrile, vortexed, and centrifuged. Each supernatant was then injected and analyzed by HPLC. The HPLC system included a Waters NovaPak C18 column (3.9 $\times$ 300mm, 4 μ M) with a mobile phase at a flow rate of 1 mL/min. A gradient was used starting with 0.2% formic acid at time 0 and reached acetonitrile/water, 70/30 v/v, at 12 min. The acetonitrile/water (70/30) mixture was maintained for 3 min (till 15 min) then the gradient was applied to reach the initial condition (0.2% formic acid) at 20 minutes. An Agilent 1100 Series sample processor with a diode array detector (Agilent model G 1315A) was used for injection and detection. HPLC peak retentions and UV/Vis spectra from samples treated by PLE were compared to those from a mixture of 3 reference compounds: UTL-5g and two prospective enzymatic products, 5-methylisoxazole-3-carboxylic acid (ISOX) and 2,4-dichloroaniline (DCA). Preliminary identification of two enzymatic products was based on comparison of both the retention times and UV/Vis spectra with those of the reference compounds.

Secondly, a different HPLC method was used to cross-check and to confirm the identities of the two enzymatic products. In this case, a Waters Symmetry C8 column (4.6 $\times$ 150 mm, 5 μ m) was used and the mobile phase parameters were as follow: Initially, 0.2 % formic acid was used as a mobile phase (isocratic at 1 mL/min) for 2 min, and a gradient was applied to reach acetonitrile/water, 70/30 v/v, at 12 min. The acetonitrile/water (70/30) mixture was maintained for 3 min (till 15 min) then the gradient was applied to reach the initial condition (0.2% formic acid) at 20 minutes. Each sample was added 1 drop of formic acid before injection. Again, the HPLC peak retentions and UV/Vis spectra were used to compare the enzymatic products with the reference compounds.

As to the enzymatic products of UTL-5g from RLE, essentially the same procedures were used to treat UTL-5g and the same HPLC approach was used to identify the enzymatic products of UTL-5g when treated with RLE.

Michaelis–Menten kinetic analysis was employed to derive the $V_{\max}$ and K_m values. Briefly, a series of UTL-5g solutions at different concentrations (0, 6.25, 12.5, 25, 50, 62.5, 75, 100, and 125 μ g/mL) were mixed individually with either porcine or rabbit esterase at 25 °C. A standard curve was established by injecting a series of standard solutions of UTL-5g. Using the slope and intercept of the standard curve, the sample peak area results were calculated and replicate samples were used in this preliminary study; the initial reaction rate (V_0) at each concentration was measured after 30 sec. Using the Lineweaver–Burk plot, $V_{\max}$ and K_m values of UTL-5g under PLE and RLE were determined individually.

3. RESULTS AND DISCUSSION

3.1. Results

Fig. 2 shows a chromatogram (at 254 nm) of a mixture of UTL-5g and its two speculated metabolites, ISOX and DCA. The wavelength of 254 nm was selected because it's between the maxima of UTL-5g and DCA. The peaks of all 3 components are well separated; distinctive UV/Vis spectra of the individual components are also shown in Fig. 2.

As shown in Fig. 3, representative HPLC chromatograms in time course show that, as UTL-5g was consumed by PLE, two major enzymatic products were generated from the enzymatic reaction and their quantities increased as the incubation time increased. Product #1 shows the same retention time and same UV/Vis spectrum as the ISOX reference (having a characteristic maximum absorption peak at 230 nm). Product #2 has the same retention time and same UV/Vis spectrum as compared to DCA (having two characteristic maximum peaks at 240 nm and 300 nm). Fig. 3 also shows that the highest concentration of product #2 at 30 min (approximately 55 μ M if calculated as DCA) is essentially the same as that of UTL-5g in the beginning, equivalent to a conversion of 1:1 mole ratio. This observation indicates that UTL-5g is a substrate and DCA is likely an enzymatic product by PLE *in vitro* although the quantity of product #1 (assuming it is ISOX) appeared lower than the 1:1 mole ratio. Therefore, both ISOX and DCA are likely the enzymatic products of UTL-5g by PLE resulted from the cleavage of the peptide bond. The lower mole ratio of ISOX as compared to UTL-5g indicates that some ISOX (by ring opening and/or oxidation) might be converted to other species that are not retained and/or observed by current method. Based on the paper from Amit S. Kalgutkar et al. [7] and classical organic chemistry, ring-opening for the ISOX in UTL-5g is not likely to happen. Therefore, it is possible that ISOX may be oxidized to become hydroxylated ISOX or related species.

Using a different HPLC column (Waters Symmetry C8, 5 μ m, 4.6 $\times$ 150mm) and a different mobile phase system, which resulted in different retention times for UTL-5g, product #1, and product #2, the same conclusion can be made. As shown in Fig. 4, both the retention time and UV spectrum of product #1 are the same as those from ISOX. The same holds true for product #2 as compared to DCA. Therefore, it can be confirmed that, under the treatment of PLE *in vitro*, the peptide bond in UTL-5g was cleaved and the major enzymatic products of UTL-5g are ISOX and DCA. This conclusion is similar to our previous report on a closely related analog of UTL-5g, UTL-5b (Fig. 1), in that a peptide bond was cleaved in a similar manner albeit it was cleaved by rat microsomes [18].

Treatment of UTL-5g by esterase from rabbit liver, based on the same HPLC approach, also showed that the same enzymatic products, ISOX and DCA, were produced *in vitro*. (Chromatograms are available as reference materials). Based on Michaelis-Menten kinetics, a Lineweaver-Burk plot was made (Fig. 5) and the results showed that $V_{\max}$ was 3.49 mM/min and K_m was 2.07 mM of UTL-5g under PLE; $V_{\max}$ and K_m of UTL-5g under RLE were 0.66 mM/min and 0.37 mM. The results indicate that UTL-5g has a better affinity to RLE than to PLE.

3.2. Discussion

Results from this work on esterase enzymatic products of UTL-5g, coupled with our previous metabolite study on UTL-5b [18], indicate that changing from the molecular scaffold of 5-methylisoxazole-4-carboxamide (e.g., leflunomide) to 5-methylisoxazole-3-carboxamide (e.g., UTL-5b, and -5g) can make a significant difference in their metabolic behaviors. The former yielded a cleaved isoxazole ring by microsomal enzymes while the latter yielded a hydrolyzed peptide bond by esterase. We also conducted an additional test which showed that when UTL-5g was incubated in mouse serum for 12 min at ambient

temperature, the same two metabolites were observed. The chromatogram is available upon request as a reference material. The result also indicates that porcine/rabbit esterase might be useful for preliminary metabolic investigation.

The difference in these two molecular scaffolds could also play an important role in their respective toxicological and pharmacological properties. For example, leflunomide has a demonstrated hepatotoxicity [19, 20], while UTL-5g protects liver from cisplatin/radiation-induced damage [21, 22]; leflunomide inhibits dehydroorotate dehydrogenase (DHODH) and is potentially teratogenic, while UTL-5b does not inhibit DHODH [23].

The results in this work also showed that UTL-5g has a better affinity to RLE than to PLE. This is not unusual because substantial species differences in esterase activities were observed among mouse, rat, dog, monkey, and human and such species differences must be considered when using these preclinical species to optimize the pharmacokinetic properties of ester compounds intended for human use [24]. As an additional note, an esterase normally hydrolyzes an ester into an acid and an alcohol but is not often reported to hydrolyze a peptide bond, which is more stable than an ester bond. As reported by Kourist et. al., a peptide bond in arylaliphatic acetamide may be cleaved by an esterase, but it needs to be under an environment that provides an intermediate hydrogen-bond network [25]. Thus our observation, cleavage of a peptide bond by porcine/rabbit esterase, is interesting and worthy of reporting.

3.3. Conclusion

In summary, based on a simple HPLC approach, it was concluded that treatment of UTL-5g by esterase (from porcine or rabbit liver) resulted in the cleavage of the peptide bond in UTL-5g and produced ISOX and DCA *in vitro*. In addition, the K_m value of UTL-5g under RLE is higher than that under PLE indicating that the affinity of UTL-5g to RLE is higher than that to PLE. Since esterase is only one of the many enzymes in liver, not exactly equal to microsomal enzymes, further work is warranted to investigate the microsomal treatment of UTL-5g in order to have a better understanding about the metabolites and the metabolic behavior of UTL-5g *in vivo*.

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Highlights

- UTL-5g is a novel small-molecule chemoprotector/radioprotector.
- As a prelude to investigating the metabolites, we set out to identify the enzymatic products of UTL-5g by esterase.
- A simple HPLC approach was used to identify the enzymatic products of UTL-5g under the treatment of porcine esterase.
- Two HPLC methods were used to compare the enzymatic products with speculated compounds and confirm their identities.
- Further, the same approach was tested using rabbit liver esterase to treat UTL-5g and the same results were found.

Fig. 1.
Structures of UTL-5b, leflunomide, UTL-5g and the corresponding enzymatic products.

(a) HPLC chromatogram of ISOX, DCA, and UTL-5g

(b) UV/Vis spectrum of ISOX

(c) UV/Vis spectrum of DCA

(d) UV/Vis Spectrum of UTL-5g

Fig. 2.

(a) HPLC chromatogram (254 nm) of a mixture of 3 reference materials (UTL-5g, ISOX, and DCA) using a Waters NovaPak C18 (4 μ m 3.9 $\times$ 300 mm) and UV/Vis spectra of ISOX (b), DCA (c), and UTL-5g (d).

(a) Incubation time = 0

(e) Time = 2 min, UV/Vis spectrum of UTL-5g

(b) Incubation time = 2 minutes

(f) Time = 2 min, UV/Vis Spectrum of product #1

(c) Incubation time = 6 minutes

(g) Time = 2 min, UV/Vis spectrum of product #2

(d) Incubation time = 30 minutes

Fig. 3.

HPLC chromatograms (254 nm) of UTL-5g treated by porcine esterase at different time points (0, 2, 6, and 20 min) and UV/Vis spectra of UTL-5g (e) and the 2 enzymatic products (f) and (g). The weak peak at 4.4 min is due to the mobile phase gradient from acid to acetonitrile/water 70/30.

(a) HPLC chromatogram (254 nm) of ISOX, DCA, & UTL-5g

(c) UV/Vis spectrum of ISOX

(b) HPLC chromatogram (at 254 nm) of UTL-5g treated by PLE at 15 min. label #1 #2

(d) UV/Vis spectrum of DCA

(e) UV/Vis Spectrum of UTL-5g

Fig. 4.

Cross-checking of the metabolites of UTL-5g by a different HPLC method
Reference materials (UTL-5g, ISOX, and DCA) were mixed and a different HPLC method (using Waters Symmetry C8 5 μ m 4.6 $\times$ 150mm) to cross-check the identities of the enzymatic products of UTL-5g by esterase. The HPLC chromatogram (254 nm) of a mixture of 3 reference materials (UTL-5g, ISOX, and DCA) and 3 UV/Vis spectra are shown as (a), (c), (d), and (e). Fig. 4 (b) shows the HPLC chromatogram of UTL-5g treated by PLE at 15 min producing products #1 and #2. Both the retention times and UV/Vis spectra (not shown) of products #1 and #2 match those of the standards respectively.

(a)

(b)

Fig. 5. Lineweaver-Burk Plot of $1/[S]$ vs $1/V_0$ for UTL-5g Incubated with porcine liver esterase (a) and with rabbit liver esterase (b)