UTL-5g Lowers Elevated Blood Levels of TNF-α and TGF-β and Increases Survival Rates in Animals Treated with LPS/D-(+)-galactosamine

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Abstract

N-(2,4-dichlorophenyl)-5-methyl-1,2-oxazole-3-carboxamide (UTL-5g) is a small-molecule chemoprotector against cisplatin and radioprotector against radiation. To further investigate its protective effects, we evaluated whether UTL-5g protects mice in a septic shock animal model. The two metabolites of UTL-5g, 5-methylisoxazole-3-carboxylic acid (Isox) and 2,4-dichloroaniline (DCA) were also evaluated side-by-side with UTL-5g. First, mice were pretreated with UTL-5g, Isox, and DCA before the i.p. injection of lipopolysaccharide (LPS)/D-(+)-galactosamine hydrochloride (D-Gal), respectively. Oral administration of both UTL-5g and Isox increased mouse survival while DCA did not, indicating that Isox is an active metabolite of UTL-5g while DCA is not. In the second study, mice were pretreated with UTL-5g or Isox individually by i.p. injection each at 30 mg/kg before LPS/D-Gal injection. The survival rates for both UTL-5g and Isox were better than those found for oral administration. In the third study, the same molar dose of UTL-5g and Isox by i.p. injection was used respectively and the results showed that UTL-5g had a better protective effect than Isox. In the fourth study, a protocol similar to the third study was used but blood samples were collected from the orbital plexus two hr after LPS/D-gal treatment. The results showed that UTL-5g lowered blood levels of both TNF-α and TGF-β elevated by LPS/D-gal. In summary, pretreatment of UTL-5g protected mice treated with LPS/D-Gal and the protection was related to the lowering of TNF-α and TGF-β levels elevated by LPS/D-Gal. In addition, UTL-5g appeared to be both an active drug and a prodrug wherein Isox is the active metabolite.

Keywords: UTL-5g, sepsis animal model, LPS/D-Gal, survival, TNF-α, TGF-β.
1. Introduction

Leflunomide is a disease-modifying anti-rheumatic drug (DMARD) but with significant potential side effects [1-5]. Based on a subtle but critical modification on the molecular scaffold of leflunomide, we designed, synthesized and, studied a series of UTL-5 compounds; several UTL-5 compounds have been shown to be TNF-α inhibitors and a particular compound, N-(2,4-dichlorophenyl)-5-methyl-1,2-oxazole-3-carboxamide (UTL-5g), is under preclinical development [6]. Although structurally similar to leflunomide, UTL-5 compounds have shown significantly different metabolic behaviors [7-9]. Leflunomide is metabolized by microsomes to become its major metabolite, teriflunomide (Fig. 1), by opening the isoxazole ring at the N-O bond [10]; however, UTL-5 compounds are metabolized by cleavage of the peptide bond but the isoxazole ring remains intact (Fig. 1). For example, UTL-5b is metabolized by rat microsomes to become 5-methylisoxazole-3-carboxylic acid (Isox) and 2-chloroaniline [8]; UTL-5g is quickly converted to Isox and 2,4-dichloroaniline (DCA) in the presence of esterase [7].

![Figure 1. Structures of leflunomide, UTL-5b, -5g, and their respective metabolites](image-url)

In terms of pharmacological effects, UTL-5 compounds are similar to leflunomide in that they are both anti-inflammatory and anti-arthritic [11-14]. However, UTL-5 compounds possess some protective effects which are significantly different from those of leflunomide. For example, UTL-5g reduces cisplatin-induced side effects by protecting kidney, liver, and platelets [6], thereby, increasing the tolerability of cisplatin [15]. In addition, UTL-5b and -5g reduce radiation-induced side effects in liver [16]. These pharmacological effects against chemotherapy/radiation-induced side effects are different from those of leflunomide. For example, lefunomide is associated with certain liver toxicity and FDA issued prescribing warnings about potential hepatotoxicity [17]; in many cases, aspartate transaminase (AST) and alanine transaminase (ALT) levels are elevated [18], whereas UTL-5g...
lowers AST/ALT levels elevated by cisplatin or radiation in our animal studies [6].

Therefore, it is of interest to further investigate the protective effects of UTL-5g and its metabolites. Because UTL-5g is a TNF-α inhibitor and lipopolysaccharide (LPS) is an endotoxin that promotes the secretion of pro-inflammatory cytokines including TNF-α [19, 20], we investigated whether UTL-5g and/or its metabolites protect mice from the lethal toxicity induced by LPS/D-(+)-galactosamine hydrochloride (D-Gal) in a septic shock animal model [21].

2. Materials and Methods

2.1. Materials

UTL-5g (HPLC purity >99%) was provided by 21st Century Therapeutics. Isox, DCA, and LPS were purchased from Sigma-Aldrich and used without further purification. CMC was purchased from Anhui Sunhe Pharmaceutical Excipients Co. (China). UTL-5g, Isox, and DCA were dispersed respectively in 1% carboxymethyl cellulose (CMC) for the animal treatment; UTL-5g and DCA maintained as suspensions in 1% CMC but the Isox preparation became a clear solution.

Kunming female mice (3-6 wk, 16-22 g) were purchased from the Third Army Medical University, Animal Testing Center (Chongqing, China). TNF-α Elisa kits were purchased from Ray Biotech (USA); TGF-β testing kits were purchased from DaKeWei Biotech, Beijing (China). All other reagents were purchased from Aldrich-Sigma unless otherwise specified.

2.2. Animal Study

In the first study, a total of 50 Kunming female mice were randomly divided into 5 groups: (1) vehicle control, (2) LPS/D-Gal, (3) UTL-5g and LPS/D-Gal, (4) Isox and LPS/D-Gal, (5) DCA and LPS/D-Gal. Isox and DCA were studied because they both are metabolites of UTL-5g [7]. For individual mice in groups 2-5, testing agents (UTL-5g, Isox, and DCA all at 60 mg/kg) were given by oral gavage respectively 1 hr before given LPS/D-gal (50 μg/kg and 600 mg/kg respectively) by i.p. injection.

In the second study, a total of 40 mice were used and divided into 4 groups: (1) vehicle control, (2) LPS/D-gal, (3) UTL-5g and LPS/D-Gal, and (4) Isox and LPS/D-Gal. In the control group, only vehicle was injected. For groups 2, 3, and 4, individual mice in groups were pretreated with corresponding agent 1 hr before given LPS/D-gal (50 μg/kg and 550 mg/kg respectively). Different from the first study, D-gal was reduced from 600 to 550 mg/kg; the doses of UTL-5g and Isox were reduced to one half (30 mg/kg each) and both were administered by i.p. injection. All animals were monitored for their body weights and survival.

In the third study, 6 groups of mice (10 mice/group) were used: (1) vehicle group, (2) LPS/D-gal, (3) UTL-5g (30 mg/kg or 0.11 mMole/kg) and LPS/D-gal, (4) UTL-5g (60 mg/kg or 0.22 mMole/kg) and LPS/D-gal, (5) Isox (14 mg/kg or 0.11 mMole/kg)-LPS/D-gal, (6) Isox (28 mg/kg or 0.22 mMole/kg)-LPS/D-gal. UTL-5g and Isox were administrated by i.p. injection 1hr before given 50 μg/kg LPS and 550 mg/kg D-gal by i.p. injection. Again, all animals were monitored for their bodyweights and survivals.

In the last study, Kunming female mice (5 mice/group) were treated in the manner identical to the third study, but blood samples were collected from the orbital plexus two hr after LPS/D-Gal treatment. Mouse plasma samples were prepared and stored at -70 ºC until analyzed by commercially available ELISA kits for levels of both TNF-α and TGF-β according to the manufactures’ instructions.

3. Results

3.1. Animal study #1

As shown in Fig 2a, on Day 1 (24 hr after given LPS/D-gal), in the group without pretreatment, all 10 mice died; in the group pretreated with oral UTL-5g (at 60 mg/kg), 1 mouse survived; in the group pretreated with metabolite #1 (Isox) at 60 mg/kg, 4 mice survived; in the group pretreated with metabolite #2 (DCA) at 60 mg/kg, all 10 mice died.
Figure 2. Effects of UTL-5g/Isox pretreatment on mouse survival rates
(a) Mouse survival rates following pretreatment by UTL-5g and Isox (both at 60 mg/kg) by oral gavage before LPS/D-Gal administration (50 μg/kg and 600 mg/kg); (b) Mouse survival rates following pretreatment by UTL-5g and Isox (both at 30 mg/kg) by i.p. injection before LPS/D-Gal administration (50 μg/kg and 550 mg/kg).
3.2. Animal study #2

Results from the second study (Fig. 2b), using both UTL-5g and Isox at 30 mg/kg by i.p. injection, showed that, in the group without pretreatment, 3 mice survived on Day 1 (24 hr), only 1 survived on Day 2, and maintained the same until Day 5. In the UTL-5g group, 5 mice survived on Day 1 (vs. 3 survived in LPS/D-Gal group, a 66% increase in survival rate) and 3 survived on Day 2 (vs. 1 survival in LPS/D-Gal group) and maintained the same until Day 5. In the Isox group, 4 mice survived (vs. 3 survived in LPS/D-gal group, a 33% increase in survival) on Day 1, 2 mice survived on Day 2 (vs. 1 survival in LPS/D-Gal group), and only 1 survived from Day 3 until Day 5.

As shown in Fig. 3, LPS/D-Gal treatment reduced the average body weights of mice by 7% on Day 1 and UTL-5g lessened this weight-loss effect to only 3% on Day 1. However, Isox did not lessen the weight-loss effect especially after Day 2 although Isox did increase the survival rate slightly; this could be because there was only one mouse survived after Day 2; thus the Isox data was not representative.

![Figure 3. Mouse body weight change vs. time from the second study](image)

**Figure 3.** Mouse body weight change vs. time from the second study
Body weight profiles of four groups: (1) vehicle control, (2) LPS/D-Gal, (3) UTL-5g + LPS/D-Gal, and (4) Isox + LPS/D-Gal. UTL-5g and Isox were both given at 30 mg/kg by i.p. injection. Each data point represents the average of 10 mice on Day 0 and lower numbers (survivals) from Day 1 and after. Standard deviations are not shown to make it easier to distinguish the profiles among these four groups.

3.3. Animal study #3

Results from the third study (Fig. 4) showed that UTL-5g protection of animals from the lethal toxicity was dose dependent in that 5/6 mice survived by UTL-5g pretreatment at 0.11/0.22 mMole/kg on Day 1 whereas only 3 mice survived without pretreatment; the survival increase was dose dependent. Fig. 4(a) shows that at a lower dose (0.11 mMole/kg), UTL-5g has a better protective effect as compared to Isox. Fig. 4(b) shows that at 0.22 mMole, UTL-5g also has a much better protective effect and resulted in higher survivals.

As to the body weights, Fig. 5 shows that the average weight of the mice in the LPS/D-Gal group was the lowest as compared to all other groups and its nadir is on Day 2. At 0.11 mMole/kg (Fig. 5a), UTL-5g group did not lose
weight and started to gain weight from Day 2. At 0.22 mMole/kg (Fig. 5b), UTL-5g group did lose weight but started to recover from Day 2. At both doses (Fig. 5a and 5b), mice in Isox group lost some weights on Day 1 and 2, but started to gain back from Day 3.

Figure 4. Survival rates with the pretreatment of UTL-5g and Isox at the same molar dose by i.p. injection (a) UTL-5g and Isox both at 0.11 mMole/kg (UTL-5g at 30 mg/kg vs. Isox at 14 mg/kg); (b) UTL-5g and Isox both at 0.22 mMole/kg (UTL-5g at 60 mg/kg vs. Isox at 28 mg/kg).
3.4. Animal study #4

As shown in Fig. 6, the final study demonstrated that TNF-α levels elevated by LPS/D-Gal (from 471 to 1782 ng/mL) were lowered by both UTL-5g and by Isox; essentially the same effects by UTL-5g and by Isox were observed for TGF-β although the reduction of TGF-β by Isox at 28 mg/kg was less than that at 14 mg/kg. In addition, the reductions of both TNF-α and TGF-β are generally parallel to the mouse survival pattern in the previous study.

Figure 5. Average body weights for mice with and without pretreatment of UTL-5g and Isox
(a) comparison of the weight loss profiles of Control, LPS/D-Gal, UTL-5g at 30 mg/kg and Isox at 14 mg/kg (equal to UTL-5g in molar conc); (b) comparison of the weight loss profiles of Control, LPS/D-Gal, UTL-5g at 60 mg/kg and Isox at 28 mg/kg (equal to UTL-5g in molar concentration)

Figure 6. Plasma levels of TNF-α and TGF-β in mice with and without pretreatment of UTL-5g/Isox
Gp 1, vehicle control; Gp 2, LPS/D-Gal; Gp 3, UTL-5g 30 mg/kg + LPS/D-Gal; Gp 4, UTL-5g 60 mg/kg + LPS/D-Gal; Gp 5, Isox 14 mg/kg + LPS/D-Gal; Gp 6, Isox 28 mg/kg + LPS/D-Gal. For both TNF-α and TGF-β levels, p < 0.05 for Gp 2 vs. Gp 4. For Fig 6a, n = 5 for all groups; for Fig 6b, n = 5 for Gp 2 & 6, n = 4 for Gp 1, 3&4, and n = 3 for Gp 5 due to insufficient plasma available.
4. Discussion

Results from the first animal study showed that both UTL-5g and Isox protected mice from the lethal toxicity induced by LPS/D-gal, but DCA had no protective effect against the lethality. The ability of both UTL-5g and Isox to increase mouse survival could be due to TNF-α inhibition at least in part because similar protective observations were reported previously for a structurally similar TNF-α inhibitor, UTL-5b [14]. In addition, UTL-5g is known to be quickly metabolized to produce Isox [7]. Therefore, it was not surprising to see that Isox also showed significant protective effect. However, in this first study, more animals survived under the treatment of Isox than under UTL-5g. This could be due to two additive factors: (1) Although the doses were the same, both at 60 mg/kg, the difference in molecular weights of UTL-5g and Isox (271 vs. 127) makes the effective molar concentration of Isox > 2 times higher than that of UTL-5g; (2) Although both UTL-5g and Isox were prepared in 1% CMC, UTL-5g stayed as a suspension whereas Isox was completely dissolved in 1% CMC; this could have resulted in a much lower oral bioavailability of UTL-5g as compared to Isox. Thus in the second animal study, both UTL-5g and Isox were administered by i.p. injection instead of oral administration.

Results from the second study again indicate that both UTL-5g and Isox protected mice treated with LPS/D-gal, but UTL-5g was more potent than Isox by i.p. injection at the same dose of 30 mg/kg. This observation supports our hypothesis that UTL-5g is both an active drug and a prodrug with Isox as the active metabolite of UTL-5g. We also used body weight as a general toxicity marker; indeed, UTL-5g lessened the weight-loss effect from LPS/D-Gal indicating its general protective effect against the toxicity induced by LPS/D-Gal. However, Isox did not lessen the weight-loss effect significantly.

The third animal study (Fig. 4) showed that UTL-5g protection of animals from the lethal toxicity (shown by the survival increase) was dose dependent. Although Isox also showed protective effect against the lethality induced by LPS/D-Gal, Isox was not as effective as UTL-5g at the same molar concentration further indicating that UTL-5g is likely both an active drug and a prodrug. Thus, it may be advantageous to use UTL-5g rather than Isox as a potential protective agent. As to the body weights, at 0.11 mMole/kg (Fig. 5a), UTL-5g group did not lose weight and started to gain weight from Day 2. At a higher dose of 0.22 mMole/kg (Fig. 5b), UTL-5g might have some negative effect itself in protecting body weight. Overall, UTL-5g and Isox both showed protective effect in body weight but UTL-5g is more effective.

The final study demonstrated that plasma levels of both TNF-α and TGF-β elevated by LPS/D-Gal were lowered by either UTL-5g or Isox although the reduction of TGF-β by Isox was not statistically significant. In addition, the reductions of both TNF-α and TGF-β are generally parallel to the mouse survival pattern in the previous study. This is logical because LPS-induced up-regulation of TGF-β receptor is associated with TNF-α expression and blockade of TGF-β signaling reduces the production of TNF-α [22]. In addition, elevated TNF-α levels induced by LPS are known to be associated with animal lethality [14, 23]. The only odd observation is that Isox at 14 mg/kg seemed to have a little better effect as compared to Isox 28 mg/kg, which can be seen in both the survival rates and the lowering of TGF-β levels, however, the difference is not statistically significant and could be partially due to experimental deviations.

It has been reported that both TNF-α and TGF-β levels are elevated in patients with sepsis [24-26]. In addition, blockade of TGF-β signaling significantly reduces the production of TNF-α by LPS-stimulated human monocyte-derived macrophages [22]. The present work on UTL-5g is in line with these reports and indicates that UTL-5g may be worthy of further investigation as a potential agent for sepsis.

This current study further expands the protective effects of UTL-5g in addition to our previous studies which showed that UTL-5g is chemoprotective and radioprotective. This work shows that UTL-5g increases the survival rates of mice treated by LPS/D-gal and its protective effects are related to the reduction of TNF-α/
TGF-β levels elevated by LPS/D-Gal. This is consistent with what we observed previously in the protective effect of UTL-5g against the toxicity of cisplatin in that elevated TNF-α levels were also lowered by UTL-5g [6]. Furthermore, our previous studies showed that a closely related TNF-α inhibitor, UTL-5b, significantly suppresses three genes that are relevant to the TNF-α pathway: Janus kinase 3 (JAK3), mitogen-activated protein kinase kinase kinase 2 (MAP3K2) and LPS-induced TNF-α factor (LITAF) [13, 14]. Based on the almost identical structural similarity between UTL-5g and UTL-5b, we believe that the mechanism of UTL-5g may be similar although the theory remains to be further verified.

Because Isox is an active metabolite of UTL-5g, it can be concluded that UTL-5g is also a prodrug. This conclusion is further supported by the facile conversion of UTL-5g into Isox [7]. However, Isox is not as potent as UTL-5g based on molecular concentration indicating that UTL-5g is also an active drug.

In summary, coupled with the chemoprotective effects of UTL-5g against cisplatin-induced side effects in kidney, liver, and platelets, as well as the radioprotective effects of UTL-5g in liver and lung, this study expands the protective range of UTL-5g potentially to LPS related diseases including sepsis [27, 28] and other immune-mediated diseases [29-31].

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References


