A Novel Leflunomide Analog, UTL-5b (GBL-5b), Suppresses JAK3, MAP3K2, and LITAF Genes

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Abstract

UTL-5b (GBL-5b) is a novel analog of leflunomide with anti-inflammatory and antiarthritic effects. It has been shown to lower serum tumor necrosis factor- α (TNF- α) level induced by lipopolysaccharide (LPS) in an animal model. In this study, the effect of UTL-5b on nitric oxide (NO) and dihydroorotate dehydrogenase (DHODH) was investigated. Our *in vitro* studies showed that (1) UTL-5b is a stronger inhibitor of NO production as compared to leflunomide and its active metabolite, teriflunomide, and (2) Unlike leflunomide, a potent inhibitor of DHODH, UTL-5b does not inhibit DHODH activity. These findings show that UTL-5b acts in a manner different from that of leflunomide. To further investigate the mode of action of UTL-5b, an *ex vivo* gene array study was performed. C57BL/6 mice were injected subcutaneously with of UTL-5b 24 hr before injection of *E. coli* LPS. Mice were sacrificed 90 min later and the whole spleen mRNA was isolated for gene microarray analysis. The results showed that UTL-5b significantly suppressed three genes that are relevant to the TNF- α pathway: Janus kinase 3 (JAK3), mitogen-activated protein kinase kinase kinase 2 (MAP3K2) and lipopolysaccharide-induced TNF- α factor (LITAF). In summary, our results showed that UTL-5b has a stronger inhibitory effect on NO production than leflunomide; yet, unlike leflunomide, UTL-5b does not inhibit DHODH *in vitro*. In addition, gene array analysis showed that the biological effects of UTL-5b are attributed at least in part to the suppression of JAK3, MAP3K2 and LITAF gene expression.

Keywords: DHODH, leflunomide, LITAF, gene array, nitric oxide, TNF- α , UTL-5b.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that causes progressive joint destruction and deformities [1, 2]. Without treatment most patients with RA become severely disabled. Although there is no cure for RA, disease modifying anti-rheumatic drugs (DMARDs), such as methotrexate, sulphalazine and leflunomide, are effective in slowing joint destruction and stabilizing joint function. Some RA patients, however, do not respond to these first-line DMARDs treatment and side effects caused by DMARDs are not uncommon in these patients.

Leflunomide (Arava®) (Figure 1) is one of the most effective isoxazole-containing heterocyclic DMARDs approved by the US Food and Drug Administration (FDA) for treating RA in 1998 [3]. Leflunomide is clinically efficacious, but it is by no means free from adverse side effects. Its sluggish clearance from the body mainly results from a very long plasma half-life (18 days) of its active metabolite, teriflunomide (previously A77 1726), and often causes adverse effects such as teratogenicity, liver toxicity, pneumonia and allergic reactions [4-6]. To improve the overall performance of this compound, a number of leflunomide analogs and teriflunomide have been synthesized and investigated by several investigators [7-9].

Recently we reported a novel analog of leflunomide, UTL-5b (also referred to as GBL-5b, Figure 1), which is anti-inflammatory in a carrageenan-induced edema animal model and anti-arthritic in a collagen-induced arthritis animal model [10]. UTL-5b showed a better anti-inflammatory effect *in vivo* as compared to leflunomide. In addition, UTL-5b has a much lower acute toxicity than leflunomide in animal studies. Further investigation showed that UTL-5b lowered the elevated serum levels of TNF- α induced by lipopolysaccharide (LPS) and D-galactosamine, and increased the survival rates of mice [10]. However, unlike leflunomide, UTL-5b did not show a statistically significant reduction of interleukin-1- α (IL-1 α) indicating that the mode of action of UTL-5b may be different from that of leflunomide.

Figure 1. Structures of leflunomide, teriflunomide, UTL-5b, and -5d.

Among other modes of action, leflunomide works by inhibiting nitric oxide (NO) and dihydroorotate dehydrogenase (DHODH; E.C. 1.3.3.1) [11, 12]. Overproduction of NO is involved in various pathological conditions including tissue damage and inflammation, RA, colitis, and septic shock [13-16]. Previous studies have shown that NO is an important regulator of TNF- α production [17-19]. For example, Yan et al. showed that endogenously produced NO can up-regulate TNF- α production in human phagocytes. On the other hand, Wang et al. showed that NO increases TNF- α production in differentiated human U937 monocytic cells by decreasing cyclic AMP. Leflunomide and its active metabolite inhibit NO *in vitro* and *in vivo* [20, 21]. DHODH is the fourth enzyme in the pyrimidine *de novo* synthesis pathway; some

researchers suggested that the immunosuppressive effects of leflunomide are closely associated with the inhibition of DHODH, which causes a profound reduction in the pyrimidine nucleotide level [22, 23]. Unfortunately, inhibition of DHODH is also related to the teratogenicity and may be a critical concern in new drug development [24].

To further investigate the potential modes of action of UTL-5b, we set to conduct *in vitro* studies to compare UTL-5b and leflunomide with regard to the inhibition on NO and DHODH. In addition, an *ex vivo* gene array study was conducted to examine the suppression of genes by UTL-5b in mice stimulated with bacterial LPS.

2. Materials and Methods

2.1 Chemicals and Reagents

UTL-5b, leflunomide and teriflunomide (All >99% HPLC purity) were provided by Dr. An-Rong Lee, National Defense Medical Center (NDMC), Taipei, Taiwan. E. coli lipopolysaccharide (LPS) was obtained from Sigma-Aldrich (St. Louis, MO). Stock solution of LPS is dissolved in PBS (1 mg/mL) and diluted to desired concentrations as needed in culture medium. Griess reagent (0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide, 2.5% H₃PO₄ in water) was purchased from Cayman Chemical (Ann Arbor, MI). Recombinant human DHODH is a gift from Prof. Lizbeth Hedstrom (Brandeis University, Waltham, MA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2.2 Cell Culture

RAW264.7 mouse macrophage cell line (identical to ATCC number: TIB-71) was obtained from Bioresource Collection and Research Center, Taiwan. Cells were routinely grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum and 10% penicillin-streptomycin according to the conventional procedures as detailed previously [25, 26]. The cells were cultured in 80 cm² tissue culture flasks (NUNC A/S, Roskilde, Denmark) and passed medium renewal every 3 days.

2.3 Microsomes

Rat liver microsomes were generously provided by Dr. Ute Kent, Department of Pharmacology, University of Michigan (Ann Arbor, MI). Liver microsomes were prepared from male Fisher 344 rats and rats treated with various inducers [27].

2.4 Animals

C57/BL6 mice were obtained from Jackson Lab (Bar Harbor, Maine). Animal studies were in full compliance with the Institutional Animal Care and Use Committee (IACUC) guidelines for the care and management of laboratory animals.

2.5 Detection of NO production

Murine macrophage RAW264.7 cells (10⁶ cells/well) were cultured in 1 mL DMEM containing 10% fetal calf serum, penicillin (100 units/mL) and streptomycin (100 µg/mL) in 24-well tissue culture plate. Cells were incubated at 37°C under 5% CO₂ and saturated water vapor. LPS solution (final concentration = 1 µg/mL) and the UTL-5b test solutions (final concentration ranging from 1, 25, 50, 100, and 200 µM) were added to individual cell cultures and placed in the incubator for 24 hours. The production of NO by RAW264.7 cells was determined by measuring the accumulated levels of nitrite in culture supernatants with the Griess reagents according to manufacturer's procedure. Briefly, 100 µL of the culture medium from each well was removed and placed in a 96-well microplate. To each well, 100 µL of Griess reagent was added and the mixtures were incubated at room temperature for 10 min. Absorption was measured by a Dynatech ELISA reader at 550 nm. NaNO₂ was used as the standard to calculate the nitrite concentration. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay was carried out to evaluate cell viability on the cell cultures by the standard method [28, 29]. Cells without treatment of the test compounds were used as control.

2.6 Inhibition of DHODH activity

Control and induced rat liver microsomes were prepared from male Fisher 344 rats and rats treated with pyridine, pregnenolone-16- α -carbonitrile, β -naphthoflavone, phenobarbital and

dexamethasone to induce different classes of cytochromes P450 in microsomes [30]. Cytochrome P450 concentrations were determined from carbon monoxide difference spectra [31]. To generate cytochrome P450 metabolites, leflunomide or UTL-5b were incubated with microsome preparations so that the final concentration of cytochrome P450 was either 250 or 750 nM. Incubations were carried out in 0.1 M potassium phosphate buffer, pH 7.4, at 37 °C in a shaking incubator. Reactions were initiated by adding NADPH (1.2 mM) to the mixtures.

Recombinant human DHODH was used for inhibition of DHODH activity *in vitro*. DHODH used in this study was a construct that had the N-terminal hydrophobic tail deleted to facilitate solubility. DHODH was assayed in Tris-HCl buffer (50 mM, pH 8.0) containing 1 mM dihydroorotate (DHO) and 1 mM Q₀ (2,3-dimethoxy-5-methyl-1,4-benzoquinone) as substrates [32]. The decrease in quinone absorbance at 400 nm for individual samples was monitored using a spectrophotometer (Shimadzu UVPC2501) at 25 °C. Activities were calculated from the slope of the tangent of the absorbance curve at the starting time.

2.7 Gene Array Study

To study the effect of UTL-5b on regulatory gene pathways relevant to TNF- α mediated inflammatory events, an *ex vivo* gene array analysis was conducted as follows: Four C57/BL6 mice were injected subcutaneously with either 100 μ l vehicle (control) or 100 μ l suspension of UTL-5b (60 mg/kg). After 24 hours, all mice were injected *i.p.* with 10 μ g of *E. coli* LPS in 50 μ l of sterile saline. Animals were monitored continuously throughout the experimental period. Mice were sacrificed by CO₂ narcosis after 90 minutes. After assurance of death by cervical dislocation, the spleen was removed by dissection and placed in a solution to stabilize mRNA. Nucleic acid preparation followed immediately. RNA isolation was conducted following the quality control protocols including Agilent Bioanalyzer testing. Gene array was conducted at Wayne State University Core Facility using the Affymetrix® GeneChip® technology. A GeneChip® Fluidics Station 450s was used to control the washing and

staining of the GeneChip arrays to insure uniformity of treatment for all samples. The GeneChip® 3000 scanner was used to scan the high resolution arrays, and the integrated autoloader maintained a controlled environment ensuring assay integrity. Data was provided by the facility indicating gene activity as 'Present,' 'Absent,' or 'Marginal.' This classification was utilized for initial results screening to determine genes that were active in control mice and suppressed by UTL-5b treatment. Numerical chip signal data was then used to determine the percent suppression of activity due to UTL-5b activity.

3. Results and Discussion

3.1 Inhibition of NO production by UTL-5b

RAW264.7 cells were treated with increasing doses of UTL-5b (0 – 200 μ M) followed by LPS stimulation in cultures for 24 hr. Accumulated levels of nitrite in culture supernatants were then determined using the Griess reagents. As shown in Table 1, the IC₅₀ values of UTL-5b were much lower than that of either leflunomide or teriflunomide. This study shows that UTL-5b is a more potent inhibitor of NO production as compared to either leflunomide or teriflunomide. Viability test shows that RAW264.7 cells were not significantly affected by the addition of the test compounds (at concentrations up to 200 μ M) and LPS by MTT assay. Cell viability for cells treated with leflunomide, teriflunomide and UTL-5b were 89, 85 and 85%, respectively, 24 hr after treatment. Thus, the inhibition of NO production by UTL-5b was not caused by the toxic effect of UTL-5b. This finding is consistent with previous *in vivo* study in which we showed that UTL-5g has a LD₅₀ of >2,000 mg/kg in animal acute toxicity test [8, 10]. The low toxicity of UTL-5b indicates that UTL-5b can be administered orally at higher doses for extended periods.

Nitric oxide (NO) plays an important role in the pathogenesis of RA [20]. In a previous study, Reddy et al. showed that treatment with leflunomide inhibits NO production in patients with RA. Their results showed that inhibition of NO synthesis may be one of the mechanisms responsible for the immunomodulatory activity of leflunomide [21]. In addition, other studies have

shown that NO increases TNF- α production [17-19]. Thus, the anti-inflammatory and anti-arthritis effects of UTL-5b are, at least in part, mediated

through the inhibition of NO and TNF- α production [10].

Table 1. Effects of UTL-5b on the NO levels in RAW264.7 treated with LPS

Compound	IC ₅₀ (μ M)	Cell viability (%) at nitrite IC ₅₀
Leflunomide	298.0 $\pm$ 10.1*	89
Teriflunomide	175.3 $\pm$ 4.7*	85
UTL-5b	17.4 $\pm$ 0.4*	85

RAW264.7 cells (10⁶/mL) were treated with increasing doses of UTL-5b, leflunomide and teriflunomide followed with LPS (1 μ g/mL) one hr later. The production of NO was determined by measuring the accumulated levels of nitrite in culture supernatants with the Griess reagents 24 hr later. Cell viability was tested with MTT colorimetric assay. IC₅₀ (μ M) shown are mean $\pm$ S.E., derived from 4-6 independent experiments of each concentration, (* P<0.05)

Table 2. Effect of UTL-5b and rat liver microsomes-treated UTL-5b on DHODH activity

Compound	Cytochrome P450 (nM)	Microsome Enzyme Inducer	Rate (μ M/min)
None	750	β -Naphthoflavone	193 $\pm$ 65*
UTL-5b (1 μ M)	750	β -Naphthoflavone	264 $\pm$ 12**
Leflunomide (1 μ M)	750	β -Naphthoflavone	2.8 $\pm$ 2.6**
UTL-5b (10 μ M)	0	-	257
UTL-5b (10 μ M)	250	Control (uninduced)	226
UTL-5b (10 μ M)	250	Phenobarbital	258
UTL-5b (10 μ M)	250	Pyridine	261
UTL-5b (10 μ M)	250	Dexamethasone	258
UTL-5b (10 μ M)	250	Pregnenolone-16- α -carbonitrile	272
UTL-5b (10 μ M)	250	β -Naphthoflavone	296

Recombinant human DHODH was assayed in Tris-HCl buffer (50 mM, pH 8.0) containing 1 mM dihydroorotate and 1 mM Q₀ (2,3-dimethoxy-5-methyl-1,4-benzoquinone) as substrates. The decrease in quinone absorbance at 400 nm for individual samples was monitored using a spectrophotometer (Shimadzu UVPC2501) at 25 °C. Activities were calculated from the slope of the tangent of the absorbance curve at the starting time. Data are means $\pm$ S.D. * n = 4, ** n = 2

3.2 Inhibition of DHODH

The active metabolite of leflunomide, teriflunomide, is a potent inhibitor of DHODH [11, 12, 23, 24, 33]. To further analyze the UTL-5b mechanism of action, we examined the effect of UTL-5b on the inhibition of DHODH. Treatment of recombinant human DHODH *in*

vitro with UTL-5b (1 μ M and 10 μ M) alone did not inhibit the enzyme activity (Table 2). To examine whether UTL-5b is a prodrug, we treated UTL-5b with either control or induced rat liver microsomes by aerobic incubation *in vitro*. Treatment of UTL-5b with these microsomes did not generate an active inhibitor of human DHODH

either. In contrast, incubation of leflunomide with microsomal preparation readily generated a potent inhibitor (presumably teriflunomide) of DHODH *in vitro* (Table 2) [33]. This finding clearly shows that UTL-5b acts in a manner different from that of leflunomide. Our result does not preclude the possibility that UTL-5b was metabolized by microsomal P450 enzymes. However, if it was, the metabolites of UTL-5b by P450 enzymes did not interfere with or inhibit DHODH. The fact that

UTL-5b is not a pyrimidine synthesis inhibitor makes it a much safer potential agent for long-term treatment of patients with RA. Previous studies have shown that leflunomide is a potent immunosuppressant drug displaying teratogenicity effect in animal studies. The teratogenicity effect of leflunomide appears to be associated with the inhibition of DHODH [24, 34]. Thus, long term treatment with UTL-5b is not expected to be associated with teratogenicity.

Table 3. Gene expression suppressed upon treatment with UTL-5b

Affymetrix ID	RefSeq ID	Gene Symbol	Gene Title	Inhibition (%)
1419679_at	NM_015771	<i>Lats2</i>	large tumor suppressor 2	88.8
1420735_at	NM_008076	<i>Gabrr2</i>	gamma-aminobutyric acid receptor	82.0
1451034_at	NM_007565	<i>Zfp36l2</i>	zinc finger protein 36	71.2
1460310_a_at	NM_008117	<i>Gh</i>	growth hormone	64.8
1429294_at	NM_027182	<i>Trip13</i>	thyroid hormone receptor interactor 13	63.6
1450771_at	NM_010243	<i>Fut9</i>	fucosyltransferase 9	63.2
1422065_at	NM_030599	<i>Nkrp1f</i>	killer cell lectin-like receptor subfamily B 1F	54.9
1452572_at	NM_009793	<i>Camk4</i>	calcium2+ calmodulin-dependent protein kinase IV	51.7
1460117_at	NM_010589	<i>Jak3</i>	JAK3 tyrosine-protein kinase	41.9
1448355_at	NM_019429	<i>Prss16</i>	protease, serine, 16	38.8
1421775_at	NM_010184	<i>Fcer1a</i>	Fc receptor, IgE, high affinity I, alphapolypeptide	38.2
1420584_at	NM_008868	<i>Pla2g2c</i>	phospholipase A2, group IIC	35.4
1449319_at	NM_138683	<i>R-spondin</i>	thrombospondin type 1	34.3
1446518_at	NM_009071	<i>Rock1</i>	serine/threonine-specific protein kinase	34.1
1429739_a_at	NM_019574	<i>Zfp278</i>	zinc finger protein 278	30.8
1423312_at	NM_011627	<i>Tpbp</i>	trophoblast glycoprotein	30.4
1422250_at	NM_011946	<i>Map3k2</i>	mitogen activated protein kinase kinase kinase 2	27.8
1421988_at	NM_011864	<i>Papss2</i>	3-phosphoadenosine 5-phosphosulfate synthase 2	25.6
1416303_at	NM_019980	<i>Litaf</i>	LPS-induced TNF-alpha factor	21.6
1453231_at	NM_183294	<i>Cdkl1</i>	cyclin-dependent kinase-like 1 (CDC2-related kinase)	15.5

C57/BL6 mice were treated with UTL-5b (60 mg/kg) or vehicle by *i.p.* injection for 24 hr. Thereafter, they were stimulated with one single *i.p.* injection of E. coli LPS (10 µg/50 µL). Ninety min later, animals were sacrificed and the whole spleen cells were used for mRNA preparation and microarray analysis. Gene array was carried out using the Affymetrix® GeneChip® technology.

3.3 Gene Array Study

Mice were treated with UTL-5b (60 mg/kg) or vehicle by *i.p.* injection. Twenty-four hr later, they were stimulated with one single *i.p.* injection of *E. coli* LPS (10 µg/50 µL). Animals were sacrificed 90 min later and whole spleen cells were used for mRNA preparation and microarray analysis. A total of 45,102 genes were analyzed. Array data was analyzed to identify genes that were active in control mice and mice treated with UTL-5b. A total of 283 genes (0.63%) fitted these criteria, however only 62 (0.14%) were sequences coding for expressed proteins. Many of the affected proteins were cytoplasmic kinases, including CDC2-related kinase, cytoplasmic tyrosine kinase, and serine/threonine-specific protein kinase, which may indicate general changes due to UTL-5b therapy (Table 3). Among them, three proteins that appear relevant to the TNF pathway were suppressed. These are: LPS-induced TNF- α factor (LITAF), mitogen activated protein kinase kinase kinase 2 (MAP3K2) and JAK3 tyrosine-protein kinase (JAK3) [35-38]. The relative suppression of these genes in UTL-5b treated animals were 21.6%, 27.8% and 41.9%, respectively (Table 3). The data suggest that a relatively small number of genes coding for proteins that are active following LPS exposure (or constitutively expressed) are affected by treatment with UTL-5b. This implies that a fairly narrow mechanism of action may exist for UTL-5b. Since we have *in vivo* data that suggests UTL-5b acts via a modulation of TNF- α levels, it is useful to observe that a few genes relevant to the TNF- α pathway were suppressed in this experiment.

MAP kinase kinase kinase (MAP3K or MEKK) is a serine/threonine-specific protein kinase involved in the MAP kinase signaling pathway. MAP3K has been shown to directly phosphorylate and activate I κ B kinases, and thus plays a role in NF- κ B signaling pathway. Schmidt et al showed that MAP3K is important in controlling the delayed activation of NF- κ B in response to stimulation with the cytokines TNF- α and IL-1 α [39]. On the other hand, Means et al. showed that transcriptional activation of the TNF- α gene in response to LPS requires the MAP kinase signaling pathways in selected macrophage populations [37]. Thus, as expected, suppression of

MAP3K gene by UTL-5b can lead to the inhibition of TNF- α production. JAK3 is also recognized as a TNF receptor-associated factor (TRAF) family protein, which might imply a possible receptor-mediated mechanism for UTL-5b. For example, Sareila et al reported that treatment of macrophages with JAK3 inhibitor, WHI-P154, inhibits human and murine iNOS expression and NO production induced by bacterial endotoxin [38]. Our data from microarray provide a possible explanation for the inhibition of NO production by UTL-5b through the suppression of JAK3 gene expression. Interestingly, these results are similar to our previous study on another leflunomide analog, UTL-5d (Figure 1), in that both JAK3 and MAP3K2 genes were suppressed by 70% and 40% respectively [40]. Even though gene array provides only a snap shot, the significant suppression of JAK3 gene by two very similar TNF- α inhibitors, UTL-5b and -5d, indicates that the direct action of UTL-5b is closely related to the upstream pathway of JAK3 gene [41].

One of the transcription factor genes down-regulated by UTL-5b treatment is LITAF. Bolcato-Bellemin et al. showed that LITAF play a crucial role in regulating the expression of TNF- α gene. They showed that upon LPS stimulation, LITAF expression is markedly up-regulated in RAW264.7 macrophage cell lines and in embryonic stem (ES) cells-derived macrophages lacking one copy of LITAF gene, TNF- α gene expression is markedly reduced [42]. Myokai et al. showed that inhibition of LITAF mRNA expression in THP-1 cells resulted in a reduction of TNF- α transcript [35]. Our findings provide the evidence that the inhibition of TNF- α by UTL-5b may be related to the suppression of LITAF gene expression.

In conclusion, UTL-5b is a novel anti-inflammatory and anti-arthritis TNF- α inhibitor that works differently from leflunomide. UTL-5b is a potent inhibitor of NO production. Yet, unlike leflunomide, UTL-5b does not inhibit DHODH *in vitro* indicating the lack of potential teratogenic toxicity. The gene array analysis suggested that a mechanism of action via LITAF, MAP3K2 or JAK3 is supported for UTL-5b. In addition, UTL-5b has a lower acute toxicity (LD₅₀ > 2,000 mg/kg) as compared to leflunomide or its active metabolite teriflunomide (LD₅₀ = 250 or 200

mg/kg, respectively) [43]. Therefore, UTL-5b is worthy of further development as a potential DMARD for the treatment of RA.

Abbreviation

DHODH, dihydroorotate dehydrogenase; DMARDs, disease modifying anti-rheumatic drugs; JAK3, Janus kinase 3; LPS, lipopolysaccharide; LITAF, LPS-induced TNF- α factor; MAP3K2, mitogen-activated protein kinase kinase 2; NO, nitric oxide; RA, rheumatoid arthritis; TNF- α , tumor necrosis factor alpha.

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