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Phosphoproteome and transcription factor activity profiling identify actions of the anti-inflammatory agent UTL-5g in LPS stimulated RAW 264.7 cells including disrupting actin remodeling and STAT-3 activation

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ABSTRACT

UTL-5g is a novel small-molecule TNF-alpha modulator. It reduces cisplatin-induced side effects by protecting kidney, liver, and platelets, thereby increasing tolerance for cisplatin. UTL-5g also reduces radiation-induced acute liver toxicity. The mechanism of action for UTL-5g is not clear at the present time. A phosphoproteomic analysis to a depth of 4943 phosphopeptides and a luminescence-based transcription factor activity assay were used to provide complementary analyses of signaling events that were disrupted by UTL-5g in RAW 264.7 cells. Transcriptional activity downstream of the interferon gamma, IL-6, type 1 Interferon, TGF-β, PKC/Ca^{2+} and the glucocorticoid receptor pathways were disrupted by UTL-5g. Phosphoproteomic analysis indicated that hyperphosphorylation of proteins involved in actin remodeling was suppressed by UTL-5g (gene set analysis, FDR < 1%) as was phosphorylation of Stat3, consistent with the IL-6 results in the transcription factor assay. Neither analysis indicated that LPS-induced activation of the NF-kB, cAMP/PKA and JNK signaling pathways were affected by UTL-5g. This global characterization of UTL-5g activity in a macrophage cell line discovered that it disrupts selected aspects of LPS signaling including Stat3 activation and actin remodeling providing new insight on how UTL-5g acts to reduce cisplatin-induced side effects.

1. Introduction

UTL-5g is a novel small-molecule TNF-α modulator with several beneficial pharmacologic effects. For example, UTL-5g reduces cisplatin-induced toxicity by protecting kidney, liver, and platelets, thereby increasing the tolerance of mice for cisplatin (Shaw et al., 2013). UTL-5g increases the survival rates of mice treated with lipopolysaccharide (LPS) (Zhang et al., 2014) and reduces radiation-induced liver damage (Shaw et al., 2012). Given the critical role for macrophages in inflammation we hypothesized that UTL-5g exerts a primary anti-inflammatory effect in vivo by suppressing macrophage activation. In support of this, UTL-5g inhibits LPS-stimulated PGE2 production in mouse RAW 264.7 cells by more than 50% (Shaw, 2015). In addition, an analog of UTL-5g blocks LPS induced NO production in RAW 264.7 cells (Shaw et al., 2011). No studies have examined the mechanism by which UTL-5g disrupts those inflammatory processes.

LPS initiated signaling is required for the immunological response to Gram negative pathogens. LPS from many bacterial species will initiate acute inflammatory responses in mammals that are typical of the host reaction to infection and immune cell responses to LPS exposure is a tool to investigate immune responses (Xie et al., 1994). The mouse RAW 264.7 cell line, derived from macrophage/monocyte tumor cells, is a common model for studying LPS-induced inflammation. RAW 264.7 cells produce a battery of mediators and proinflammatory cytokines when exposed to LPS, and the paradigm of LPS treatment of cultured RAW cells is used extensively to investigate the mechanisms of action for anti-inflammatory compounds (Chiang et al., 2005; Kim et al., 2007).

Genome- and system-scale technologies are valuable tools for mechanism discovery as they enable novel and unanticipated findings (Coombs et al., 2012). The expanding capabilities of mass spectrometry based phosphoproteomics in terms of depth of coverage and sample

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multiplexing (Erickson et al., 2015; Sharma et al., 2014), are making it a powerful systems-level approach for mechanism of action studies. It has already been applied to mechanism of action determination for inorganic mercury (Caruso et al., 2014; Caruthers et al., 2014), deoxyvinylinol (Pan et al., 2013), ammonia (Harder et al., 2014), and to identify the target profiles of kinase inhibitors (Li et al., 2010; Pan et al., 2009). It has emerged from these studies and from other investigations of compounds with known mechanisms (Pines et al., 2011) that the phosphoproteome can provide a more precise determination of mechanism of action than transcriptome or total proteome analysis. Further, because LPS signaling is mediated by a well characterized cascade of protein phosphorylation, phosphoproteomic analysis is a natural choice to investigate disruptions of its action.

A major outcome of LPS stimulation of RAW 264.7 cells is an increase in transcription of genes whose products mediate inflammatory responses. In order to capture both the dynamics of the phosphorylation cascade and its transcriptional outcome, which comprise the main components of LPS signaling in macrophages, we applied a combination of phosphoproteomics and transcription factor activity analysis to examine the mechanism by which UTL-5g suppresses LPS activation of RAW cells.

2. Materials and methods

2.1. Chemicals and reagents

Lipopolysaccharide (LPS, *E. coli* 0111:B4) was obtained from Sigma-Aldrich (St. Louis, MO). M-PER protein extraction reagent, phosphatase inhibitors and Opti-MEM® were purchased from ThermoFisher Scientific (Waltham, MA). Dulbecco's modified eagle medium (DMEM), bovine calf serum (BCS), fetal bovine serum (FBS), non-essential amino acids (NEAA) and penicillin/streptomycin were obtained from (HyClone, Logan, UT). Cignal Finder Immune Signaling Pathway Reporter Arrays and Attractene transfection reagent were products of QIAGEN (Valencia, CA). Dual-Luciferase reporter assay system was purchased from Promega (Madison, WI). RAW (264.7) mouse macrophage/monocytic cell line was acquired from ATCC (Manassas, VA). They were routinely grown in DMEM supplemented with 10% heat inactivated BCS. Anti-p-JNK (T183/Y185) rabbit polyclonal antibody and peroxidase-labeled goat anti-rabbit secondary antibody were obtained from R & D Systems (Minneapolis, MN). BCA protein assay reagent was purchased from Pierce (Waltham, MA). TiO_2 beads were obtained from Titansphere, GL Sciences (Tokyo, Japan). Tandem mass tag labeling reagents were obtained from Thermo Fisher Scientific (Waltham MA). Dithiothreitol (DTT), iodoacetamide (IAA) and Lithium dodecyl sulphate (LiDS) were purchased from Sigma (St Louis, MO). UTL-5g (Lot#1182-MEM-3D, Purity > 99%) (Fig. 1) was synthesized at Kalexsyn Medicinal Chemistry (Kalamazoo, Michigan). The polyclonal anti-p-Ser5 L-plastin antibody was a kind gift from Dr. Elisabeth Schaffner-Reckinger (University of Luxembourg) (Janji et al., 2006).

2.2. Transcription factor assay

Multi-pathway activity assays were carried out using Cignal Finder Immune Signaling 10-Pathway Reporter Arrays according to the manufacture's instruction. Transfection of RAW 264.7 cells (1×10^5/well) was performed using Attractene (0.4 µl/well) at 37 °C for 16 h. After transfection, cells were washed and replenished with assay medium (Opti-MEM® containing 0.5% of FBS, 1% NEAA, 100 U/ml penicillin and 100 µg/ml streptomycin). Thereafter, cells were treated with varying doses of UTL-5g for 60 min and then challenged with 100 ng/ml of LPS. After an additional 16 h of incubation, cells were washed and lysed. Luciferase assay was carried out with Dual-Luciferase reporter assay system and the assay was performed directly in the multi-well plate with Fluoroskan FL microplate luminometer (Thermo Fisher Scientific) at room temperature.

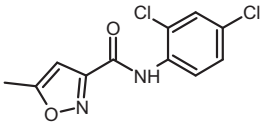

Fig. 1. Chemical structure of UTL-5g.

2.3. Western Blot analysis

For the JNK analysis, RAW 264.7 cells (2×10^6/well) were treated with UTL-5g from 0.2 to 10 µM at 37 °C for 60 min, followed with LPS 10 µl/well (100 ng/ml final) for an additional 30 min. Thereafter, cells were washed once in cold MEM and lysed with 150 µl of M-PER protein extraction reagent containing protease and phosphatase inhibitors and EDTA. The cell mixtures were vortexed gently for 10 min on ice and cell debris were removed by centrifugation at 14,000×*g* for 15 min. The sample supernatants were removed and separated by SDS-PAGE (12% gel). After transfer to a nitrocellulose membrane, the membrane was blotted with anti-p-JNK (Thr183/Tyr185) rabbit polyclonal antibody followed by peroxidase-conjugated goat anti-rabbit secondary antibody. JNK bands were visualized by enhanced chemiluminescense detection methods.

For the plastin-2 analysis RAW 264.7 cells (2×10^6/well) were treated with 10 or 50 µM UTL-5g at 37 °C for 60 min, followed with LPS 5 µl/well (50 ng/ml final) for an additional 30 min. Samples were processed as indicated for the phospho JNK analysis except that the nitrocellulose membrane was probed with anti-p-plastin (Ser5).

2.4. Sample preparation and LC-MS3

RAW 264.7 cells (1.0×10^6/dish) were grown for three days in 15 cm dishes, reaching 80% confluence ($> 3 \times 10^7$/dish) and then treated as described in the results (Section 3.2). An additional sample treated with 25 µM pervanadate for 15 min was also prepared to improve our ability to detect phosphotyrosine (pTyr). Pervanadate treatment can result in an accumulation of pTyr to up to 20% of phosphorylated residues (Caruthers et al., 2014). We reasoned that the inclusion of a pTyr enriched sample would improve our ability to detect pTyr residues in data-dependent LC-MS3 analysis. The dishes were placed on ice and harvested by scraping and then rinsed with ice-cold Hank's solution, pelleted and frozen. Cells were lysed by resuspension in 1% LiDS and heating at 95 °C for 5 min. Protein concentration in the lysates was determined using a BCA assay. Samples were reduced with DTT and alkylated with IAA and then digested overnight with trypsin at 1:50 (w/w) at a sample protein concentration of 1.0 mg/ml in buffer containing 100 mM Tris, 0.1% LiDS, and 10% acetonitrile. All samples were evaluated by SDS-PAGE to ensure full digestion before proceeding to phosphopeptide isolation. Phosphopetides were isolated using 5 µm TiO_2 beads at a ratio of beads to protein of 8:1 (w/w). Eluted peptides were dried, resolubilized and evaporated again to remove ammonium from the TiO_2 procedure. Samples were then resolubilized in buffer for TMT labeling, which was carried out according to the manufacturer's instructions. The individual samples were then pooled for analysis. The pooled, labeled peptides were fractionated using an SCX MicroSpin Column (Harvard Apparatus, Holliston MA) with elution in 7 fractions using 5–125 mM ammonium formate. Each fraction was dried, resolubilized in 0.1% FA and submitted for LC/MS3 analysis. Peptides were loaded onto a 75 µm × 25 cm, Acclaim PepMap 100 column and eluted into a Thermo Fusion Tribrid mass spectrometer (Thermo Fisher Scientific) by a gradient of acetonitrile from 2% to 30% over 110 min. The mass spectrometer was set to conduct multinotch MS3 analysis to limit interference from coeluting peptides (McAlister et al., 2014). The top 10 most abundant peptides in each MS1 scan were selected for MS2 fragmentation using CID at 32 to