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Effects of a pyrrole-based, microtubule-depolymerizing compound on RAW 264.7 macrophages

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ABSTRACT

RAW 264.7 murine macrophages were exposed to the pyrrole-based compound 3,5-Dibromo-4-(3,4-dimethoxyphenyl)-1H-pyrrole-2-carboxylic acid ethyl ester (JG-03-14), which is a known microtubule depolymerizing agent with antitumor activity [1,2,3]. In this study exposure to JG-03-14 reduced the production of pro-inflammatory molecules by macrophages activated with lipopolysaccharide (LPS). Treatment with the pyrrole-based compound decreased the concentration of tumor necrosis factor-α (TNF-α) and nitric oxide (NO) released from the macrophages. Exposure to JG-03-14 also decreased TNF-α mRNA expression levels and the protein expression levels of inducible nitric oxide synthase (iNOS), the enzyme responsible for NO production in the activated macrophages. Furthermore, JG-03-14 treatment significantly changed the degradation profile of IκB-β, an inhibitor of the NF-κB transcription factor, which suggests that JG-03−14 may attenuate the activation of the LPS-induced NF-κB signaling pathway needed to produce the pro-inflammatory mediators. We conclude that JG-03-14 possesses anti-inflammatory properties.

1. Introduction

Studies of 3,5-Dibromo-4-(3,4-dimethoxyphenyl)-1H-pyrrole-2-carboxylic acid ethyl ester (JG-03-14, Fig. 1), a tetrasubstituted brominated pyrrole, have demonstrated that this compound possesses potent microtubule depolymerizing properties by binding tubulin at the colchicine site [1–4]. As a major component of the eukaryotic cytoskeleton, microtubules contribute to cell physiology by participating in chromosome segregation during mitosis, organelle trafficking and cell signaling events, and disruption of tubulin dynamics is the target of several anticancer drugs. Although colchicine was the first drug shown to bind tubulin, resulting in the loss of microtubules and the prevention of new microtubule formation, it is too toxic to be used in a clinical setting [4–6]. Therefore, less toxic compounds, such as JG-03-14, that bind at the colchicine site of tubulin have drawn considerable attention. Earlier studies that demonstrated the importance of the C-2 and C-4 positions of JG-03-14 for tubulin binding also showed that the tubulin-depolymerizing property of this compound correlated with anti-proliferative activity in a wide range of cancer cell lines [2,3]. Further investigations demonstrated that JG-03-14 promotes autophagic cell death of tumor cells and that it is active against tumor cells expressing the multidrug resistance pump, providing additional support for the potential use of JG-03-14 to treat malignancies [7,8]. However, little is known about the effect of JG-03-14 on immune cells, specifically macrophages, which detect invading microorganisms in the tissues, carry out phagocytosis, and produce pro-inflammatory mediators [9]. While acute inflammation is protective against infections and tissue injury, chronic inflammation may lead to inflammatory disorders and cancers

Abbreviations: NF-κB, Nuclear factor kappa B; LPS, Lipopolysaccharide; TLR4, Toll-like receptor 4; IKK, Inhibitor of nuclear factor kappa B kinase; IκB-α, Inhibitor of nuclear factor kappa B alpha; IκB-β, Inhibitor of nuclear factor kappa B beta; TNF-α, Tumor necrosis factor alpha; iNOS, Inducible nitric oxide synthase; NO, Nitric oxide; MTOC, Microtubule organizing center.

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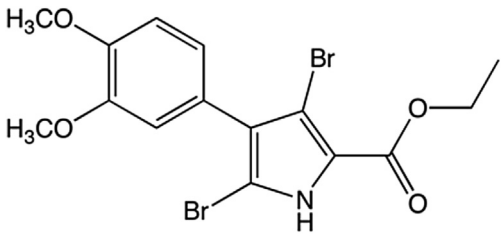

(JG-03-14)

Fig. 1. Structure of 3,5-Dibromo-4-(3,4-dimethoxyphenyl)-1H-pyrrole-2-carboxylic acid ethyl ester (JG-03-14).

[10–14]. Studies have shown that other pyrrole-containing compounds possess anti-inflammatory properties [15–17]. Therefore, in addition to understanding the microtubule depolymerizing properties of JG-03-14, investigating the effects of JG-03-14 on the pro-inflammatory activity of macrophages is important in order to expand our knowledge of the compound's bioactive properties and its clinical potential.

The Nuclear Factor-kappa B (NF-κB) transcription factor is one of the most important regulators of the inflammatory response. In macrophages, the pro-inflammatory NF-κB signaling pathway can be activated by lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, which binds to the Toll-like receptor 4 (TLR4) complex and triggers the production of pro-inflammatory molecules [9]. Initiation of this pathway involves a complex signaling architecture that ultimately phosphorylates and activates the IKK complex, which in turn phosphorylates the inhibitory proteins IκB-α and IκB-β that are bound to the NF-κB transcription factor [18]. These inhibitory proteins are then ubiquitinated and degraded in a cytosolic proteasome [18]. The now-active NF-κB translocates into the nucleus and promotes the transcription of inflammatory response genes. In mice, some of these genes include *TNFA*, which encodes the cytokine tumor necrosis factor-α (TNF-α) and *NOS2*, which encodes inducible nitric oxide synthase (iNOS). The iNOS enzyme catalyzes the reaction of L-arginine to citrulline, which produces nitric oxide (NO) radicals as a byproduct, useful as both a vasodilator and an anti-pathogenic oxidant.

To assess the effects of JG-03-14 on the macrophage inflammatory response, our study investigated how exposure to JG-03-14 affected the production of NO, iNOS, and TNF-α by RAW 264.7 macrophages activated with LPS. We also investigated JG-03-14's effects on the relative mRNA expression of TNF-α as well as the degradation of the IκB-β inhibitory protein after LPS activation. We found that JG-03-14 suppressed the production of the inflammatory molecules and our data further suggest that this may be caused by an effect on the NF-κB signaling pathway.

2. Materials and methods

2.1. Cell culture

RAW 264.7 macrophages were purchased from ATCC and plated in T75 filter-top flasks, where they were managed under sterile conditions and grown at 37 °C with 5% CO_2. Cells were maintained in RPMI complete medium containing 10% fetal calf serum and supplemented with L-glutamine, non-essential amino acids, MEM vitamins, and penicillin/streptomycin and grown to approximately 75–95% confluence. Cell populations were activated with LPS (E. coli 055:B5; Sigma–Aldrich). Cell lysates and supernatants were harvested 2.5 min–2 h after LPS-activation for IκB-β experiments, 4 h after activation for TNF-α experiments, and 20 h after activation for NO/iNOS studies. The JG-03-14 compound was provided by Dr. John Gupton and solubilized in DMSO, and then diluted in fresh medium (to < 1% DMSO) before being added to culture media. In all experiments, JG-03-14 compound was administered 1 h before LPS activation. After LPS incubation, cell culture supernatants were collected. Adherent cells were then washed twice with phosphate buffered saline (PBS), and homogenized with lysis buffer consisting of 0.05 M Tris buffer (pH 7.5), 0.3 M NaCl, 2 mM EDTA, 0.5% Triton-X 100, 2 μg/mL Leupeptin (Sigma–Aldrich), 1 μg/mL Aprotinin (Sigma–Aldrich), and 0.2 mM PMSF (Sigma–Aldrich). Both supernatants and lysates were stored at −20 °C until assayed.

2.2. Fluorescence microscopy

Cells were plated on coverslips held in 6-well plates and allowed to adhere overnight. After exposure to JG-03-14 and a 4-h LPS activation period, cells were washed with PBS, fixed with paraformaldehyde for 10 min, washed with 0.5% Triton-X in PBS for 5 min, and then placed in a donkey serum blocking solution for 30 min. Cells were then incubated in 1 ug/mL mouse anti-α-tubulin antibody by placing coverslips facedown on a parafilm surface in the antibody diluted in the block solution for 30 min. The coverslips were then washed in PBS for 5 min four times. Cells were then incubated in 1:4000 dilution of DAPI, 1:1000 dilution of phalloidin, and 1:10,000 dilution of donkey anti-mouse antibody conjugated to ALEXA 488 for 30 min. The coverslips were again washed in PBS for 5 min four times. Coverslips were then mounted onto microscope slides in glycerol mounting media and sealed with clear nail polish. Images were acquired using an Olympus IX-83 microscope outfitted with a PLAN APON 60x/1.42NA DIC objective, an EXFO mixed gas light source, Sutter filter wheels and shutters, a Hamamatsu ORCA-Flash 4.0 V2 sCMOS camera, and Metamorph imaging software. Z-stack images (0.2 μm steps) were captured sequentially using the Sedat Quad filter-set (Chroma), and exposure times were maintained constant within an experimental data set.

2.3. MTT cell viability assay

MTT compound (Sigma–Aldrich) was solubilized in PBS in limited light conditions at a concentration of 5 mg/mL and left overnight at 4 °C to dissolve completely. Populations of 5.0×10^3 cells were added to wells in a 96-well flat-bottom microtiter plate and were allowed to adhere for 2 h before media was aspirated and replaced with 100 μL fresh media. Cells were then administered JG-03-14 and underwent a 20-h incubation. Next 10 μL of the MTT solution were added to each well in limited light conditions and allowed to incubate in the dark for 4 h in the CO_2 incubator. The formazan crystals were solubilized with 100 μL of isopropanol in 0.04 M HCl. Absorbance was immediately read at 570 nm using a Beckman–Coulter DTX 800 Multimode Detector. It is also important to note that for all experiments performed in this study microscopic examination did not detect any noticeable differences in the viability of cultures, regardless of treatment (data not shown).

2.4. Nitric oxide assay

Populations of 3.3×10^6 cells were incubated with LPS for 20 h before being harvested for NO assessment. Nitrite accumulation in the supernatants was assessed using a standard Greiss assay. Briefly, an equal volume of supernatant (50 μL) was reacted with equal volumes of each Greiss reagent (50 μL each) and the absorbance was read at 550 nm using a Beckman–Coulter DTX 800 Multimode Detector.