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Biaryl amide compounds reduce the inflammatory response in macrophages by regulating Dectin-1

Kyeong Eun Hyung [a,1], Mi Ji Lee [a,1], Yun-Jung Lee [a], Do Ik Lee [b], Hye Young Min [b], So-Young Park [c], Kyung Hoon Min [b,*], Kwang Woo Hwang [a,**]

[a] Host Defense Modulation Laboratory, College of Pharmacy, Chung-Ang University, 221 Heukseok-dong, Dongjak-gu, Seoul 156-756, Republic of Korea
[b] College of Pharmacy, Chung-Ang University, 221 Heukseok-dong, Dongjak-gu, Seoul 156-756, Republic of Korea
[c] Pharmacognosy Laboratory, College of Pharmacy, Dankook University, San#29, Anseo-dong, Dongnam-gu, Cheonan-si, Chungnam 330-714, Republic of Korea

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ABSTRACT

Macrophages are archetypal innate immune cells that play crucial roles in the recognition and phagocytosis of invading pathogens, which they identify using pattern recognition receptors (PRRs). Dectin-1 is essential for antifungal immune responses, recognizing the fungal cellular component β-glucan, and its role as a PRR has been of increasing interest.

Previously, we discovered and characterized a novel biaryl amide compound, MPS 03, capable of inhibiting macrophage phagocytosis of zymosan. Therefore, in this study we aimed to identify other biaryl amide compounds with greater effectiveness than MPS 03, and elucidate their cellular mechanisms.

Several MPS 03 derivatives were screened, four of which reduced zymosan phagocytosis in a similar manner to MPS 03. To establish whether such phagocytosis inhibition influenced the production of inflammatory mediators, pro-inflammatory cytokine and nitric oxide (NO) levels were measured. The production of TNF-α, IL-6, IL-12, and NO was significantly reduced in a dose-dependent manner. Moreover, the inflammation-associated MAPK signaling pathway was also affected by biaryl amide compounds. To investigate the underlying cellular mechanism, PRR expression was measured. MPS 03 and its derivatives were found to inhibit zymosan phagocytosis by decreasing Dectin-1 expression. Furthermore, when macrophages were stimulated by zymosan after pretreatment with biaryl amide compounds, downstream transcription factors such as NFAT, AP-1, and NF-κB were downregulated.

In conclusion, biaryl amide compounds reduce zymosan-induced inflammatory responses by downregulating Dectin-1 expression. Therefore, such compounds could be used to inhibit Dectin-1 in immunological experiments and possibly regulate excessive inflammatory responses.

1. Introduction

Macrophages are archetypal innate immune cells that perform various functions, including phagocytosis, lymphocyte activation through antigen presentation, synthesis of antimicrobial agents, and production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and IL-1β [1].

Innate immune recognition relies on an array of germ-line encoded receptors generally referred to as pattern recognition receptors (PRRs) [2]. PRRs recognize conserved pathogen motifs known as pathogen-associated molecular patterns (PAMPs), which are not normally present in the host. This recognition process therefore allows host immune cells to distinguish between self and non-self antigens. Upon PAMP binding, PRRs induce intracellular signaling essential in inducing an initial immune response, ultimately leading to the removal of the invading foreign antigens. PRRs are divided into four families: Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors, retinoic acid-inducible gene 1-like receptors, and C-type lectin-like receptors (CLRs) [3,4].

Dectin-1 was the first CLR to be discovered, and is the most studied to date [5]. It is capable of recognizing several fungal species through binding of the PAMP β-glucan. Zymosan is a yeast-derived particle composed predominantly of β-glucan and mannan, and is widely used as a cellular activator for investigating various phagocytic responses [6]. Upon binding particulate β-glucans, Dectin-1 can induce diverse

* Correspondence to: K.H. Min, College of Pharmacy, Chung-Ang University, 221 Heukseok-dong, Dongjak-gu, Seoul, Republic of Korea.
** Correspondence to: K.W. Hwang, Laboratory of Host Defense Modulation, College of Pharmacy, Chung-Ang University, 221 Heukseok-dong, Dongjak-gu, Seoul, Republic of Korea.
E-mail addresses: khmin@cau.ac.kr (K.H. Min), khwang@cau.ac.kr (K.W. Hwang).
[1] These authors contributed equally to this work.

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cellular responses, including actin-mediated phagocytosis, activation of respiratory burst through production of reactive oxygen species (ROS), T lymphocyte activation via antigen presentation, and production of pro-inflammatory cytokines and lipid mediators, contributing to the removal of fungal cells [3,5,7,8].

Phagocytosis is the actin-dependent uptake of targets greater than 0.5 μm in diameter [9] and is a crucial cellular process, both during homeostasis and upon infection or tissue damage. Receptors on the surface of professional phagocytes bind to targets either directly or indirectly [10]. Dectin-1 is a key PRR for the recognition and phagocytosis of β-glucans, and stimulation of this receptor alone is sufficient to trigger phagocytosis [11]. Moreover, transfection of Dectin-1 into 3T3, fibroblasts not normally capable of phagocytosis, enables these cells to phagocytize β-glucan [12]. Thus, Dectin-1 is a bona fide phagocytic receptor, and macrophages expressing Dectin-1 are considered professional phagocytes.

Previously, through screening of a chemical library, we identified the biaryl amide compound MPS 03 and demonstrated its effective regulation of macrophage zymosan phagocytosis [13]. In the current study, we screened derivatives of this compound to identify biaryl amide molecules demonstrating more effective modulation of zymosan phagocytosis in macrophages. Furthermore, we evaluated the effect of these compounds on the regulation of pro-inflammatory cytokines and nitric oxide (NO). We also sought to determine the mechanisms by which biaryl amide compounds regulate zymosan phagocytosis, and investigate their effect on Dectin-1 and its role in this process in macrophages.

2. Materials and methods

2.1. Biaryl amide compounds

MPS 03 and derivatives 1 to 3 were synthesized using a method previously described [13]. Compound 4 was prepared by using a similar procedure except that reaction with bromobutane instead of bromopropane. The crude product was purified by flash column chromatography. The structure of biaryl amide compound was confirmed by NMR (Supplementary data 1).

2.2. Cell culture

RAW 264.7 murine macrophage cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's Medium (Cellgro, Manassas, VA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Cellgro), 10 mM hydroxyethyl piperazineethanesulfonic acid (HEPES), 2 mM L-glutamine, and 100 U/mL penicillin and 100 μg/mL streptomycin (Cellgro), at 37 °C in a 5% CO_2 humidified incubator.

2.3. Materials

Fluorescein isothiocyanate (FITC)-conjugated zymosan (Z2841) and unlabeled zymosan (Z2850) were obtained from Molecular Probes (Eugene, OR, USA). Zymosan depleted of its TLR stimulatory activity was prepared by boiling of 250 μg zymosan in 1 mL 10 M sodium hydroxide for 30 min and washing three times with sterile PBS [14]. Cytochalasin D (cytoD; C2618) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Capture and detection antibodies used in enzyme-linked immunosorbent assays (ELISAs) for TNF-α, IL-6, and IL-12p40 were purchased from BD Biosciences (San Diego, CA, USA). MPS 03 and its derivatives were provided by Professor K.H. Min (College of Pharmacy, Chung-Ang University, Seoul, Republic of Korea).

2.4. Phagocytosis assay

RAW 264.7 cells were plated onto 24-well plates at 2×10^6 cells per well and allowed to adhere for 2 h. They were then pretreated with cytoD (2 or 10 μM) or biaryl amide compounds (20 μM) for 30 min. Following pretreatment, the cells were detached by scraping, washed, and then incubated in medium containing FITC-conjugated zymosan (5 particles per cell) for 1 h at 37 °C. After being thoroughly washed to remove unbound zymosan, the cells were resuspended in phosphate-buffered saline (PBS), and analyzed using a FACSCalibur flow cytometer and CellQuest Pro software (both BD biosciences, USA).

2.5. ELISA

RAW 264.7 cells were plated onto 96-well plates at 2×10^5 cells per well and left to adhere for 2 h before being pretreated with biaryl amide compounds (10 or 20 μM) for 30 min. After pretreatment, cells were treated with zymosan (200 μg/mL) for 24 h at 37 °C. Supernatants were then collected and the concentrations of TNF-α, IL-6, and IL-12p40 in each sample were measured by ELISA. The 96-well plates were incubated overnight at 4 °C with capture antibody, following which, the wells were washed three times with 1% PBS-Tween (PBS-T), incubated for 1 h with blocking solution at room temperature, and washed a further four times with PBS-T. Samples and diluted standards were then added to the plate and incubated overnight at 4 °C. The detection antibody was subsequently added after four PBS-T washes. Following 45 min incubation at room temperature, the wells were washed and incubated with avidin-conjugated alkaline phosphatase for 30 min, also at room temperature. The substrate solution was then added and plates were kept at room temperature for 5 to 30 min before addition of a stop buffer. The colored reaction product was measured by absorbance at a wavelength of 405 nm.

2.6. NO assay

RAW 264.7 cells were plated onto 96-well plates at 2×10^5 cells per well and allowed to adhere for 2 h. Cells were then pretreated with biaryl amide compounds (20 μM) for 30 min before being treated with zymosan (200 μg/mL) for 20 h at 37 °C. Supernatants from cell cultures were then examined for NO production. Each supernatant was mixed with an equal volume of Griess reagents (Sigma-Aldrich) and the light absorbance of this mixture at 540 nm was determined using a microplate reader (Emax, Molecular Devices). Nitrite concentration was determined using dilutions of a nitrite standard solution.

2.7. Western blot analysis

Total protein was extracted from cells in radioimmunoprecipitation assay buffer (Thermo Scientific, Daejeon, Republic of Korea). Equal amounts of protein for each sample were then separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes, and probed with antibodies against β-actin and phosphorylated forms or non-phosphorylation form of the following proteins: extracellular signal-related kinase (p-ERK), p38 (p-p38), and c-Jun N-terminal kinase (p-JNK; Cell signaling, Danvers, MA, USA). The membranes were then incubated with anti-mouse immunoglobulin G (IgG) or anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling).

Nuclear and cytoplasmic proteins were extracted using an NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific, Rockford, IL, USA), and equal amounts of protein for each sample were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked for 1 h at room temperature and incubated overnight at 4 °C with antibodies against the following proteins: lamin B1, c-Jun (AP-1), nuclear factor-κB (NF-κB), nuclear factor of activated T cells (NFAT;