International Immunopharmacology 55 (2018) 323–329

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Effects of guluronic acid (G2013) on SHIP1, SOCS1 induction and related molecules in TLR4 signaling pathway

Seyed Shahabeddin Mortazavi-Jahromi[a,c], Ali Farazmand[a,b], Nasrin Motamed[a,b], Shadi Sadat Navabi[c], Abbas Mirshafiey[c,*]

[a] Department of Cellular and Molecular Biology, Kish International Campus, University of Tehran, Kish, Iran
[b] School of Biology, University College of Science, University of Tehran, Tehran, Iran
[c] Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

ARTICLE INFO

Keywords:
G2013
Guluronic acid
SHIP1
SOCS1
TLR4
MyD88

ABSTRACT

Objective: This research aimed to study the anti-inflammatory and immunomodulatory effects of guluronic acid (G2013) on gene expression of TLR4, MyD88, SHIP1, SOCS1, NF-κB, and assessment of the level of IL-1β as a pro-inflammatory cytokine in HEK-Blue hTLR4 cell line.

Methods: The cytotoxicity of G2013 was assessed by the MTT assay. The mRNA expression levels of the mentioned genes were measured by qRT-PCR. IL-1β concentration in culture media was determined using ELISA method.

Results: MTT assay demonstrated that G2013 (before the concentration of 125 μg/ml) had no cytotoxic effect on HEK-Blue hTLR4 cells. Our results indicated that the low and high doses of this drug could significantly reduce the gene expression of TLR4 and MyD88, as compared to the control group ($p < 0.05$). Moreover, it was found that the low dose of this drug could significantly increase the gene expression of SHIP1 and SOCS1, as compared to the control group ($p < 0.05$). Furthermore, the study findings revealed that the level of NF-κB gene expression significantly reduced, in both doses of G2013 compared to the control group ($p < 0.05$, $p < 0.01$, respectively). Our data showed that the level of IL-1β in culture media decreased by both doses of this drug in comparison to control group ($p < 0.05$).

Conclusion: This study indicates that G2013 is able to induce SHIP1, SOCS1 and reduce TLR4, MyD88, NF-κB at the level of gene expression and decrease IL-1β as a pro-inflammatory cytokine which might be recommended for reduction of inflammatory reactions.

1. Introduction

Toll-like receptors (TLRs) are a large group of transmembrane proteins. Thirteen of them have been identified in humans and mice. They play a major role in pathogens patterns recognition, activation of innate and adaptive immunity, the guidance of signaling pathways [1,2]. A TLR contains an extracellular leucine-rich repeat domain. It identifies microbial components specifically and it contains the intracellular signaling domain known as Toll/interleukin-1 receptor (TIR) domain, which finally activates the activation factors and causes the production of proinflammatory cytokines [2]. TLR4 is the main recognition receptor of lipopolysaccharide (LPS), a Gram-negative bacterial cell wall component [3]. Activation of TLR4 leads to stimulation of MyD88-dependent pathway and MyD88-independent pathway [4]. These pathways regulate the balance between cell survival and inflammation. TLR signaling is controlled by some negative regulators to prevent aberrant inflammatory responses. The negative regulators include splicing variants for adapters or their related proteins, ubiquitin ligases, deubiquitinases and transcription regulators, which stop TLR signaling at separate levels [5–9].

Src homology 2 domain–containing inositol-5-phosphatase 1 (SHIP1) is a 145-kDa intracellular protein, which is encoded in human by the *INPP5D* gene [10,11]. The catalytic function of SHIP1 is the dephosphorylation of phosphatidylinositol (3, 4, 5) trisphosphate (PIP3), which is the product of phosphoinositide 3-kinases (PI3K), and convert it into phosphatidylinositol (3, 4)-bisphosphate (PIP2) [12]. SHIP1 intracellular expression increases upon the initial contact with LPS, which increases endotoxin tolerance [13]. SHIP1 negatively regulates LPS-induced of Akt downstream of PI3K. Moreover, SHIP1 inhibits LPS-induced mitogen-activated protein kinase (MAPK) activation and IκB-α degradation. Although it is independent of the PI3K pathway, it is assumed that this happens by preventing complex formation

* Corresponding author at: Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran 14155-6446, Iran.
E-mail address: mirshafiey@tums.ac.ir (A. Mirshafiey).

https://doi.org/10.1016/j.intimp.2018.01.003
Received 20 August 2017; Received in revised form 18 December 2017; Accepted 3 January 2018

between TLR4 and MyD88 [14]. SHIP1 occurs in endothelial cells, [15] and therefore can involve in the regulation of LPS signaling in the cells. PIP3 is another major molecule, which involves regulating different cell signaling, including protein kinase B/Akt, secretion of inflammatory mediators and many other pathways. These pathways regulate cell proliferation and differentiation, protein translation, apoptosis prevention, and most importantly cells migration [16,17]. Therefore, the activation of SHIP1 redirects signaling away from PIP3-mediated activation cascades including those that regulate immune activation (Akt phosphorylation) and chemotaxis in the inflammatory cells. Suppressor of cytokine signaling 1 (SOCS1) is a protein which is encoded by *SOCS1* gene in humans [18]. SOCS1-encoding mRNA is at very low levels in most cells. It down-regulates TLR signaling [19,20]. TLR4 signaling activation induces inflammatory cytokines such as IL-1β, IL-6, and TNF-α. With respect to the role of SOCS1 in inhibiting the signaling created by cytokines, one of the most significant functions of SOCS1 is to regulate the intracellular signaling activated by cytokines, which were induced by TLR's primary signaling [21,22]. Moreover, SOCS1 regulates TLR-induced NF-κB activation through direct interaction with the p65 subunit of NF-κB [23]. NSAIDs play an important role in the management of the inflammatory disease [24]. In recent years, researchers have tried to identify safer and more effective types of anti-inflammatory and immunomodulatory drugs. The guluronic acid (G2013) molecule (Fig. 1) is an agent with the low molecular weight and has less toxicity compared with other NSAIDs. G2013 is a novel designed a drug which could be classified as a non-steroidal anti-inflammatory drug (NSAID), with the immunomodulatory property [25].

The present study aimed to investigate the anti-inflammatory and immunomodulatory effects of G2013 in both low and high dose on gene expression of TLR4, MyD88, SHIP1, SOCS1, NF-κB, and assessment of the level of IL-1β as a pro-inflammatory cytokine in a transfected cell line of HEK293 (known as HEK-Blue hTLR4).

2. Material and methods

2.1. MTT cytotoxicity assay

Cell viability and cytotoxicity studies of G2013 were performed by MTT assay. The cell counting was conducted using Trypan blue and HEK-Blue hTLR4 cells were then seeded (15×10^4 cells per well) in 96-well plates. 24 h after seeding, two-fold dilutions of G2013 (at a concentration of 200 ng/ml-125 μg/ml) were added to cells and incubated overnight. Then, 100 ml of MTT solution (0.5 mg/ml) was added to each well and incubated for 4 h. The incubation was stopped by the addition of dimethylsulfoxide dissolved in formazan. Finally, absorbance was measured using a microplate reader at 570 nm.

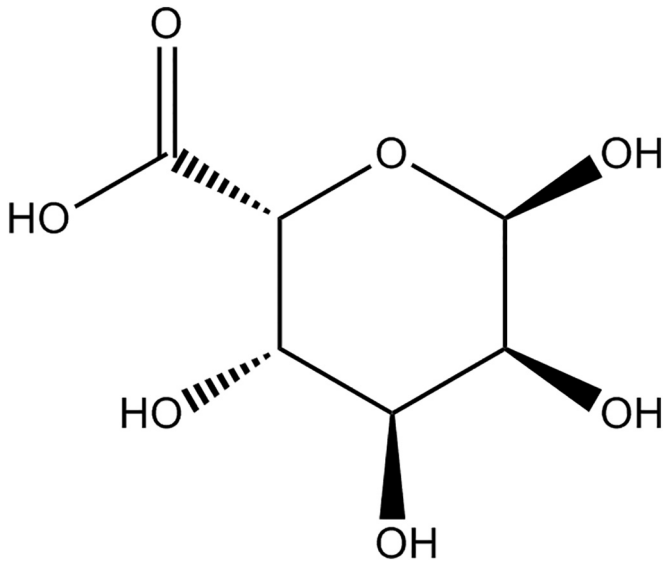

Fig. 1. The chemical structure of G2013 (guluronic acid) patented (DE-102016113017.6).

2.2. Cell line and cell culture

Engineered Human Embryonic Kidney (HEK) cell line HEK-Blue hTLR4 (the gift of M. Yousefi) was cultured in complete growth medium (Roswell Park Memorial Institute 1640 with 10% heat-inactivated fetal bovine serum, 100 units per ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate (Gibco, Life Technologies, USA) and 100 μg/ml Normocin™ (InvivoGen, USA)) in presence of cell line-specific selection antibiotics. Selected antibiotics for HEK-Bule hTLR4 cells contained 100 μg/ml Zeocin™, 200 μg/ml Hygromycin B gold and 30 μg/ml Blasticidin. The HEK-Blue-hTLR4 cells are HEK 293 cells stably expressing human TLR4 (hTLR4) receptor and the needed accessory proteins to TLR4, MD-2, and CD14. These accessory proteins interact with LPS at the TLR4 complex to induce NF-κB activation. In addition, these cells are also engineered with a reporter gene, secreted embryonic alkaline phosphatase (SEAP), which is produced following NF-κB activation. The cultures were grown in 25 cm^2 flasks at 37 °C and 5% carbon dioxide (CO_2). Cells were subcultured when they were 60–80% confluent. The media were changed two to three times a week.

2.3. Drug preparation

The G2013 as a small molecule ($C_6H_{10}O_7$) with uronic acid structure patented (DE-102016113017.6), and as a novel NSAID with the immunomodulatory property was prepared in immunology section of pathobiology department of Tehran University of Medical Sciences (TUMS).

2.4. Treatment of cells with G2013 and endotoxin

HEK-Blue hTLR4 Cells were seeded at 5×10^5 cells in 1000 μl per well of a 24-well plate and cultured in complete growth medium as described above. Twenty-four hours after seeding, cells were then treated with G2013 low dose (5 μg/well), G2013 high dose (15 μg/well), LPS-EB (LPS from *E. coli O111:B4*) (100 ng/ml), G2013 low dose + LPS, G2013 high dose + LPS and blank medium (untreated; negative control) using endotoxin-free water in quadruplicate wells, and incubated for 24 h at 37 °C in 5% CO2.

2.5. RNA extraction

RNA was extracted from control and treated cells using Hybrid-R™ Mini kit (GeneAll, South Korea) according to the manufacturer's guidelines. The extracted RNA quality was identified by electrophoresis on the GelRed™ (Biotium, USA) contained agarose gel and measured absorption at A260/280 nm by NanoDrop® ND1000 spectrophotometer (Isogen Life Science, Netherlands).

2.6. cDNA synthesis and quantitative real-time PCR (qRT-PCR)

Total RNA from control and treated cells was reverse transcribed using HyperScript™ First-strand Synthesis Kit (GeneAll, South Korea) following the manufacturer's instructions. The cDNA synthesis reactions included 10 μl total RNA, Random hexamer 1 μl, 1 μl dNTPs, 2 μl Nuclease-free water, 2 μl RT reaction buffer (10 ×), 2 μl MDTT (0.1), 2 μl HyperScript Reverse Transcriptase (200 U/μl), 1 μl ZymAll™ RNase Inhibitor. The 20 μl reactions were incubated for 5 min at 65 °C, 1 min on ice, 40 min at 50 °C, 5 min at 85 °C, and held at 4 °C. Real-time PCR was performed using SYBR® Premix Ex Taq™ II (Dalian, Takara Co., Ltd), produced cDNA and appropriate primers (Table 1). Twenty microliters of real-time PCR reactions included 1 μl genomic template