RESEARCH ARTICLE

The Immunomodulatory Role of G2013 (α-L-Guluronic Acid) on the Expression of TLR2 and TLR4 in HT29 cell line

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Abstract: Background/Objectives: The non-steroidal anti-inflammatory drugs (NSAIDs) play crucial role in the controlling of inflammatory diseases. Due to the vast side effects of NSAIDs, its use is limited. G2013 or α-L-Guluronic Acid is a new NSAID with immunomodulatory features. Considering the leading role of TLRs in inflammatory responses, in this study, we aimed to evaluate G2013 cytotoxicity and its effect on the expression of TLR2 and TLR4 molecules.

Method: HEK293-TLR2 and HEK293-TLR4 cells were cultured and seeded on 96-well cell plate, and MTT assay was performed for detecting the viability of the cells after treatment with different concentrations of G2013. HT29 cells were grown and treated with low and high doses of G2013. After total RNA extraction and cDNA synthesis, quantitative real-time PCR were performed to assess the TLR2 and TLR4 mRNA synthesis.

Results: We found that concentrations of ≤125 µg/ml of G2013 had no apparent cytotoxicity effect on the HEK293-TLR2 and -TLR4 cells. Our results indicated that after G2013 treatment (5 µg/ml) in HT29 cells, TLR2 and TLR4 mRNA expression decreased significantly compared with the untreated control group (p=0.02 and p=0.001 respectively).

Conclusion: The results of this study revealed that G2013 can down regulate the TLR2 and TLR4 gene expression and exerts its inhibitory effect. Our findings are parallel to our previous finding which showed G2013 ability to down regulate the signaling pathway of TLRs. However, further studies are needed to identify the molecular mechanism of G2013.

Keywords: G2013, α-L-Guluronic Acid, NSAIDs, TLR2, TLR4, HT29.

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1. INTRODUCTION

Toll-like receptors (TLRs) are a family of receptors located on plasma or endosome membrane...
which have a critical function in both innate and adaptive immunity [1]. These receptors sense endogenous damage-associated molecular patterns (DAMPs) as well as conserved molecules of pathogen-associated molecular patterns (PAMPs) derived from bacteria, viruses, fungi and parasites [2, 3]. TLRs recognize various components of microbial products; for example, TLR4 recognizes bacterial lipopolysaccharide (LPS), TLR2 recognizes lipoproteins and lipoteichoic acid (LTA) of bacteria; TLR5 recognizes flagellin and TLR7, TLR8 and TLR9 sense microbial RNA and DNA [4]. The engagement of TLRs by their specific ligands leads to the dimerization of the signaling domain of these receptors and initiation of downstream signaling followed by the recruitment of adaptor proteins which activates various protein kinases, leading to the activation of different transcription factors such as NF-κB and IRFs [5]. Translocation of these transcription factors to the nucleus results in the expression of inflammatory cytokines, IFN-α, IFN-β and the expression of co-stimulatory molecules [6].

Toll like receptors, especially TLR2 and TLR4 are involved in variety of autoimmune and inflammatory disorders and are related to a range of diseases such as inflammatory bowel disease (IBD), multiple sclerosis (MS), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Ankylosing spondylitis and type 1 diabetes (TID) [7, 8]. Therefore, it is suggested that the inhibition of TLR2 and TLR4 as well as their signaling pathway could be considered in treatment of these diseases.

The non-steroidal anti-inflammatory drugs (NSAIDs) are used as pain relievers and inflammation treatment [9]. The NSAIDs play main roles in the controlling inflammatory diseases such as spondyloarthritis and RA, scleroderma and SLE [10, 11, 12]. By decreasing the level of prostaglandin E2 (PGE2), they inhibit the synthesis of prostaglandins and exert their therapeutic effect. However, because of their vast side effects in gastrointestinal (GI) track, kidneys and cardiovascular system, the use of these drugs is restricted. It has been shown that indomethacin (as NSAID) can injure the small intestine via a TLR4/MyD88-dependent pathway [14]. Moreover, NSAIDs can increase TNF production and may exacerbate the pro-inflammatory condition in RA [15]. Thus, it is necessary to develop new anti-inflammatory therapeutic regimens without NSAIDs-induced complications.

G2013 or α-L-Guluronic Acid (C6H10O7) is a new NSAID with immunomodulatory features. It is a low weight molecule extracted from alginate by chemical hydrolysis technique in Immunology Department of Tehran University of Medical Sciences (TUMS). This molecule is the epimer of β-D-Mannuronic acid with established tolerability and effectiveness in animal models of experimental autoimmune encephalomyelitis (EAE), adjuvant induced arthritis (AIA), nephrotic syndrome and acute glomerulonephritis [16–19].

Before the anti-inflammatory aspects of G2013 were searched in experimental model of MS. The inflammatory cells and plaques in G2013 dosed mice were lower than control group. However, G2013 significantly reduced the serum level of nitric oxide (NO) in EAE mice that were parallel to clinical findings such as lower severity and delay in disease onset [20]. In 2016, Mirshafiey et al. searched the effect of G2013 on the several determinants of oxidative stress in Sprague–Dawley rats and found the anti-aging property for G2013 [21]. In 2017, in a Preclinical and pharmacotoxicological evaluation, the safety of G2013 following oral administration was approved [22]. Moreover, in that year, the safety of this drug on differentiation, maturation and function of immature and mature dendritic cells (DCs) was observed [23].

At the present time, different clinical trials are running to find the efficacy of G2013 in ankylosing spondylitis (IRCT201609183739N4), RA (IRCT201609283739N5) as well as MS (IRCT20161113739N6).

In this study, considering the leading role of TLRs activation in inflammatory responses and importance of G2013 as a new NSAD and immunomodulatory agent, we aimed to evaluate the G2013 cytotoxicity and its effect on the gene expression of TLR2 and TLR4 molecules.

2. MATERIAL AND METHODS

2.1. Cell Culture of HEK293 Cell Line

Cell lines of HEK293-TLR2 and HEK293-TLR4 were purchased from the National Cell Bank of Iran, Pasteur Institute of Iran. At first, HEK293 cells were cultured on 25cm² cell culture flask (biofilm, USA) in culture medium consisting of RPMI 1640 enriched with 10% heat-inactivated
fetal bovine serum (FBS), 2 mM glutamine, 1 mM sodium pyruvate, antibiotics including 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, life Technologies USA). The cells were cultured at 37°C under a humidified atmosphere in the presence of 5% carbon dioxide (CO2) until reaching the 80% of confluency. The medium was changed 3 times a week., HEK293-TLR2 and HEK293-TLR4 cells were then divided into 96-well plate in the concentration of 1×10^4 per well.

2.2. Cell Viability Test

Cell viability and cytotoxicity study of G2013 were assessed using MTT assay. After 24 hours of cell seeding into 96-well cell culture plate, serial dilutions of β-D-Mannuronic Acid (200 ng/ml to 125 µg/ml) were added to the wells and cells were incubated overnight. 100 µl of MTT color solution (0.5 mg/ml) was then added into each well and incubated for 4 hours. After finishing the incubation time, dimethyl sulfoxide was added to dissolve the formazan crystals. Finally, the optical absorbance was measured by microplate reader at 570 nm. The results were compared with the untreated cells for test control.

2.3. Cell Culture of HT29 Cell Line

HT29 was purchased from the Cell Bank at the International genetic and biological center, and grown on 25 cm² cell culture flask (biofilm, USA). Cells were maintained in RPMI enriched with 10% FBS, 20mM HEPES and L-glutamine, and 100 U/ml penicillin and 100 µg/ml streptomycin. Cell culture medium was changed every 1-2 days until after 4-5 days, full polarization of the HT-29 cell monolayer was achieved. One million HT29 cells were allowed to attach and grow in 12-well cell culture plates.

2.4. Treatment of HT29 Cells

Twenty-four hours after seeding cells, the cells were treated by low dose (5 µg/ml) and high dose (25 µg/ml) of G2013. Moreover, the cells were treated by low dose of G2013 (5 µg/ml) accompanying with 1 µg/ml LPS (Sigma, USA) and high dose of G2013 (25 µg/ml) accompanying with 1 µg/ml LPS. The cells incubated in 37°C and 10% CO2 for 20-24 hours. In this study, non-treated wells were considered as control groups for drug treated wells.

2.5. RNA Extraction and Revers Transcription (RT-PCR)

Total RNA was isolated manually from treated and non-treated cells by Qiazele (Qiagen, USA). The quality and concentration of isolated RNA were assessed by UV spectrophotometry (Nano Drop ND1000) using the 260/280 and 260/230 nm ratio, respectively. Total RNA was reverse transcribed to cDNA using reverse transcription kit (Takara, Japan), according to the company instructions.

2.6. Quantitative Real-Time PCR

The quantitative real-time PCR was done by SYBR Green PCR Master Mix (Takara, Japan). The primer pairs of housekeeping gene (β-actin) and TLR2 and TLR4 genes were as follow: β-actin: F; 5’- CGTTGACATCCGTAAGACC -3’, R; 3’- TAGAGCCACCAATCCACACA -5’, TLR2: F; 5’- TGTCTCTCACGAACC -3’, R; 3’- CAACCTCATTAAGGTGC -5’, and TLR4: F; 5’- GAGCCACCTGGACCTTCAAAATAC -3’, R; 3’-GTAGGACATGACGAGTTGATC-5’. The transcripts were quantified using the ABI Step One Plus Real-time PCR system (ABI System, USA) according to the manufacturer's instructions. Amplification conditions were: 95°C for 15 s followed by 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. The β-actin housekeeping gene was used for the normalization of amplification. The relative frequency of TLR2 and TLR4 genes was calculated by 2^{-ΔΔCt} method [24]. Amplified products were analyzed by agarose gel electrophoresis for reassuring the size of PCR products.

2.7. Statistical Analysis

The results were analyzed by SPSS software (version 16). Kolmogorov–Smirnov and Shapiro-Wilk tests were used to determine whether our data were normally distributed. Paired samples t-test was used for detecting statistical differences in parametric analysis and Wilcoxon tests were used for non-parametric analysis. P values ≤0.05 were considered statistically significant. Prism software (version 5) was used for drawing graphs.

3. RESULTS


Before more studies on the immunomodulatory properties of G2013, we assessed its cytotoxicity
using MTT assay. Several concentrations of G2013 were tested including, 200 ng/ml, 1, 5, 25 and 125 µg/ml. The result of MTT assay showed that G2013 at concentration equal or lower than 125 µg/ml had no apparent cytotoxicity effect on the HEK293-TLR2 and HEK293-TLR4 cells (Figs. 1, 2).

### 3.2. The Effect of G2013 on the Gene Expression of TLR2 and TLR4

Our results of quantitative real-time PCR assay indicated that the expression of TLR2 mRNA in HT29 cells in the low-dose G2013 treatment group was decreased significantly compared with the untreated control group, p=0.02 (Fig. 3). Moreover, the expression of TLR4 mRNA in HT29 cells which was treated with low-dose G2013 showed a significant reduction compared with control group, p=0.001. (Fig. 4).

#### Fig. (1). The percent of viability of HEK293-TLR2 cells after treatment with different concentrations of G2030 by MTT assay. The MTT assay showed that the concentrations equal or less than 125µg/ml of G2013 are associated with more than 50% of cells viability in HEK293-TLR2.

#### Fig. (2). The percent of viability of HEK293-TLR4 cells after treatment with different concentrations of G2030 by MTT assay. The MTT assay showed that the concentrations equal or less than 125µg/ml of G2013 are associated with more than 50% of cells viability in HEK293-TLR4.

#### Fig. (3). The effect of G2013 on the expression of TLR2 mRNA in HT29 cells. Abbreviations: LG: low dose of G2013 (5µg/well); HG: high dose of G2013 (25µg/well); LPS: lipopolysaccharide; Data are shown as mean±SD. The sign of * displays significant statistical difference with control group. The sign of # displays significant difference with LPS group.

#### Fig. (4). The effect of G2013 on the expression of TLR4 mRNA in HT29 cells. Abbreviations: LG: low dose of G2013 (5µg/well); HG: high dose of G2013 (25µg/well); LPS: lipopolysaccharide; Data are shown as mean±SD. The sign of * displays significant statistical difference with control group. The sign of # displays significant difference with LPS group.
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This research showed that the LPS treatment significantly up-regulated the gene expression of both TLR2 and TLR4 in HT29 compared with controls, p= 0.001 and p= 0.02, respectively, whereas, the combination of LPS with low and/or high doses of G2013 did not make any significant changes in the gene expression of TLR2 and TLR4 compared with controls.

4. DISCUSSION

The NSAIDs play a significant role in the management of inflammatory disorders [25]. Due to the side effects of these drugs, there are a lot of efforts to find an efficient and safe anti-inflammatory agents. According to the previous studies, the immunosuppressive property of G2013 was shown in experimental models and peripheral blood mononuclear cells. The inhibition of immune cells infiltration in inflammatory foci and significant reduction of the nitric oxide (NO) production were shown as molecular mechanism of therapeutic effect of G2013 in mice model of MS [26]. However, according to the lower expression of the molecules involved in TLRs signaling cascade in some studies [27-30], we aimed to find the gene expression level of TLR2 and TLR4 in G2013-treated cells.

Interestingly, we found that G2013-treated HT29 cells down regulated the expression level of TLR2 mRNA and TLR4 mRNA. This finding was parallel to the previous studies.

In 2015, Hajvalili et al. showed that the treatment of HEK-293-TLR4 cells by G2013 could suppress the TLR4 downstream signaling molecules consists of interleukine-1 receptor associated kinase-1 ( IRAK1) and tumor necrosis factor (TNF) receptor associated factor 6 (TRAF6), but there was no difference in gene expression of miR-146a as anti-inflammatory factor [27].

In 2016, Mortazavi Jahromi et al. indicated that after LPS stimulation of HEK-Blue h TLR4, G2013 could significantly reduce the gene expression of miR-155, since this miRNA contributes to several pathological processes such as inflammation and autoimmunity [28].

In 2017, Sharifi et al. showed that G2013 at concentration of 25 mg/ml (high dose) significantly down-regulated NF-κB, IκB and MyD88 mRNA expression and suppressed the secretion of IL-1β in human mononuclear cells [29]. In other study, these researchers showed that G2013 is able to reduce significantly the mean florescence intensity (MFI) of TLR2 and TLR4 as well as gene expression of NF-κB in common variable immunodeficiency (CVID) patients [30].

The results of present study accompanied by the previous studies confirm that G2013 can down-regulate the expression of TLR2 and TLR4 as well as their signaling cascade and exert its inhibitory effects. We highly recommend further studies in this area to identify the specific target and molecular mechanism of this novel agent.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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REFERENCES


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