Evaluation of the Effect of α-L-Guluronic Acid (G2013) on COX-1, COX-2 Activity and Gene Expression for Introducing this Drug as a Novel NSAID with Immunomodulatory Property

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Abstract: Background: Nonsteroidal anti-inflammatory drugs (NSAIDs) are used to treat the pathological pain and inflammation through inhibition of cyclooxygenase (COX) enzyme and disruption of the synthesis of prostaglandins (PGs). The α-L-guluronic acid (G2013) patented (PCT/EP2017/067920), as a novel NSAID with the immunomodulatory property, has been shown its positive effects in experimental models of multiple sclerosis and anti-aging.

Objective: This study was aimed to investigate the effects of G2013 on the gene expression and activity of COX-1/COX-2 enzymes in order to introduce a novel NSAID for the treatment of inflammatory diseases.

Method: The mRNA expression levels of COX-1/COX-2 were measured by qRT-PCR. The PGE2 concentration in culture media was determined using ELISA method.

Results: Our results demonstrated that the low and high dose of G2013 could significantly reduce the gene expression of COX-1 and COX-2, as compared to the control treated with LPS (p < 0.05). In addition, data showed that 5, 50 and 500 mMol/ml doses of this drug can significantly reduce activities of COX-1 and COX-2, as compared to the control treated with LPS and AA (p < 0.0001).

Conclusion: This study revealed that G2013, as a novel NSAID with the immunomodulatory property, is able to reduce the gene expression and activity of COX-1/COX-2 enzymes. According to the findings, this agent might be categorized and introduced as a novel NSAID for the treatment of inflammatory diseases.

Keywords: COX-1, COX-2, G2013, guluronic acid, immunomodulation, NSAIDs, PGE2.

1. INTRODUCTION

Cyclooxygenase (COX) enzyme, officially known as prostaglandin endoperoxide H synthase, has a significant role in the synthesis of prostanooids, including prostaglandins (PGs), prostacyclin, and thromboxane (TX) [1]. According to studies, there are two isoforms of this enzyme in mammals, namely COX-1 and COX-2, with independent genes and different patterns of expression [2, 3]. In some laboratory studies by Sighorsson et al., COX-3 was investigated; however, it was not experimentally diagnosable and identifiable, which might be due to its short lifespan [4]. Cyclooxygenases are an integral membrane, glycosylated and homodimer proteins comprising of 70-kDa subunits. Each subunit has a heme and a catalytic site and is placed in a layer of phospholipid membrane through hydrophobic surfaces of amphipathic helixes [5]. Both isoforms of COX are relatively similar in terms of size, amino acid sequence, structural shape, and enzymatic activities [6, 7]. In the enzyme pathway of COX, its two well-known isoforms (COX-1 and COX-2) initiate the synthesis of PGs, thromboxane A2 (TXA2), and other prostanooids. Production of these prostanooids is due to the presence of arachidonic acid (AA). The release of AA from membrane phospholipids is mediated by
secretive or cytoplasmic phospholipase A2 activity. When AA is secreted, the COX isoforms catalyze the formation of PGH2 from AA, which may be converted into PGE2, PGF2, PGD2, PGI2 and TXA2 in different cells and conditions [8]. The COX-1 and COX-2 genes are mapped on chromosome 9q32-q33.3 and 1q25.2-q25.3, respectively [9]. The COX-1 gene is 22 kb in length with 11 exons and no TATA box. There is limited information about the regulation of COX-1 expression. The COX-2 gene is 8 kb in length with 10 exons. It contains a TATA box and several inducible enhancer elements including NF-kb and CRE [9, 10]. The COX-1 is an innate protein with immunity function in humans; whereas, COX-2 is an inducible enzyme that suddenly increases by inflammation, stress, degenerative process, cancer, cytokines, etc. As a result, the COX-2 expression is called immediate-early response gene [11]. The nonsteroidal anti-inflammatory drugs (NSAIDs) have the analgesic, antipyretic, and anti-inflammatory effects by inhibiting COX enzyme [12]. Aspirin is the only specific inhibitor of COX-1 that forms a covalent bond with the enzyme. Acetylation of serine 530 inhibits AA from binding to the active site, leading to the irreversible inhibition of the enzyme [13]. Other NSAIDs, such as indomethacin, naproxen, and ibuprofen compete with AA to bind with the active site, resulting in reversible inhibition of COX-1 and COX-2 [14-16]. The above-mentioned drugs equally inhibit these two isozymes, in that the dosage required for reducing inflammation, the risk of stomach irritation, and risk of damage to the gastrointestinal mucosa is increased [17]. Therefore, many researchers have tried to identify safer and more effective anti-inflammatory drug.

NSAIDs have a significant role in the management of inflammatory diseases [18]. The α-L-guluronic acid (G2013) patented (PCTEP2017067920) [19], is categorized as a novel drug of NSAID family (Fig. 1). This substance with low molecular weight and anti-inflammatory and immunomodulatory properties is derived from a linear polymer of alginate, which is a major component of cell walls of brown algae and polysaccharide capsule among certain bacteria [20]. Alginate has wide applications, in pharmacy. Alginates are linear copolymers formed by β-D-mannuronic acid (M-block) and α-L-guluronic acid (G-block) linked by 1→4 glycosidic linkage [21, 22]. According to a study, in addition to its therapeutic effects, G2013 is less toxic than other NSAIDs for the gastrointestinal system and kidney function [20]. G2013 has shown its positive effects in experimental models of multiple sclerosis and anti-aging [23-25]. Moreover, Mortazavi Jahromi et al. showed that G2013, as a novel immunomodulatory drug, is able to decrease miRNA-155 expression significantly, following stimulation by LPS [26]. This study was aimed to investigate the anti-inflammatory and immunomodulatory effects of G2013 on expression and activity of COX-1/COX-2 enzymes in order to introduce a novel NSAID for the treatment inflammatory disease.

2. MATERIALS AND METHODS

2.1. Blood Collection and PBMCs Isolation

The venous blood of twenty healthy volunteers (between 25-45 years, were recruited at Blood Transfusion Center of Rafsanjan-Iran, with considering the informed consent protocol of the declaration), were collected on sodium heparin as an anticoagulant. The healthy volunteers were from non smoking group and without using illicit drugs or suffering from diabetes, hypertension or metabolic disease and they had no history of the chronic or autoimmune diseases. The Peripheral Blood Mononuclear Cells (PBMCs) were isolated from blood sample through Ficoll-Hypaque (Mediatech Cellgro, USA) density gradient centrifugation from buffy coats provided from the healthy blood. The PBMCs were then resuspended in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin, 2mM L-glutamine and 1mM sodium pyruvate (all reagents provided by Gibco, USA). The cell viability was determined by trypan blue 0.4% (Sigma-Aldrich, USA) exclusion at 1: 4 dilution (1 part of cells: 4 parts of dye). The samples were only used when viability was > 95%.

2.2. Preparation of G2013

The α-L-guluronic acid (G2013) [19] with molecular formula (C₆H₁₀O₇) and IUPAC name [(2R/3S/4S/5S)-2,3,4,5-tetrahydroxy-6-oxohexanoic acid] were prepared from alginic acid sodium salt (Sigma-Aldrich, St. Louis, MO) as a reference sample. The purification method was carried out based on a modified procedure of the acid hydrolysis method by Nazari et al [20]. Briefly, alginic acid sodium salt (100g) was dissolved gently in 20% H₂SO₄ at 0°C and the mixture was thoroughly stirred at room temperature. This solution was heated at 80°C until its color was changed from a creamy color to light brown. The hydrolysate was cooled to room temperature and precipitated by centrifugation (3700 × g). The precipitate was redissolved using 1M Na₂CO₃. The solution was then adjusted to pH 2.85 with 0.1M HCl. The precipitate was collected and washed once with distilled water. The final precipitate (α-L-guluronic acid) was spread over and dried out in Petri dishes [20]. The method was validated by characterizing the hydrolytic products using Fourier Transform Infrared (FT-IR) spectroscopy and Carbon-13 Nuclear Magnetic Resonance (¹³C-NMR) spectroscopy for confirming its molecular weight (194.139g/mol) and exact/monoisotopic mass (194.043g/mol).

2.3. Treatment of PBMCs with G2013 and LPS

The PBMCs were cultured in 4-well of culture plate with RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin, 2mM L-glutamine and 1mM sodium pyruvate (all reagents from Gibco, USA). In this way, the first well contains 2 ×10⁶ cells in the absence of lipopolysaccharide (LPS) and G2013.
Table 1. Primer Sequences Used in this Experiment.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Accession Number</th>
<th>Primer Sequences</th>
<th>PCR Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-1</td>
<td>NM_000962.3</td>
<td>F: 5'-TGTGACCTCCCTTCTACTACCC-3'</td>
<td>157</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-CTCCTGCCCTCTCTCTCTGCTG-3'</td>
<td></td>
</tr>
<tr>
<td>COX-2</td>
<td>NM_000963.3</td>
<td>F: 5'-CTTCTCCTGTCGTGCTGTGATGAT-3'</td>
<td>199</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-GCCCTGCCCTATGTCTGTCTGCT-3'</td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>NM_0001101.3</td>
<td>F: 5'-CTTCTCCTGTCGTGCTGTGATGAT-3'</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-GCCCTGCCCTATGTCTGTCTGCT-3'</td>
<td></td>
</tr>
</tbody>
</table>

F: forward; R: reverse.

(negative control) and second well contains $2 \times 10^6$ cells in the presence of LPS-EB (LPS from E. coli O111: B4, Sigma-Aldrich, USA) (1µg/ml) in order to stimulate PBMCs and absence of G2013 (positive control). The third and fourth wells of culture plate contain $2 \times 10^6$ cells and LPS-EB (1µg/ml), so that after 4h incubation at 37°C, we added 2.5 mMol/ml of G2013 (the low dose of the test well) and 12.5 mMol/ml of G2013 (the high dose of the test well) to these wells, respectively. Cells were incubated for 18 h at 37°C in humidified 5% CO2 incubator.

2.4. RNA Extraction

The RNA was extracted from control and treated cells using Hybrid-R™ Mini kit (GeneAll, Republic of Korea) according to the manufacturer’s guidelines. The integrity and concentration of total RNA were identified using a NanoDrop® ND1000 spectrophotometer (Isogen Life Science, The Netherlands).

2.5. cDNA Synthesis and Quantitative Real-Time PCR (qRT-PCR)

The total RNA from control and treated cells were reverse transcribed using HyperScript™ First-strand Synthesis Kit (GeneAll, Republic of Korea) and the manufacturer’s instructions. The cDNA synthesis reactions included 10µl total RNA, 1µl Random hexamer, 1 µl dNTPs, 2µl Nuclease-free water, 2µl RT reaction buffer (10x), 2µl MDTT (0.1), 2µl HyperScript Reverse Transcriptase (200U/µl), 1µl Zy-mAll™ RNase Inhibitor. The total mentioned agents (20µl) were incubated for 5min at 65°C, 1min on ice, 40min at 50°C, 5min at 85°C, and held at 4°C. Real-time PCR was performed using SYBR® Premix Ex Taq™ II (Dalian, Ta-kara Co., Ltd), produced cDNA and appropriate primers (Table 1). Twenty microliters of real-time PCR reactions included 1µl genomic template DNA, 10µl SYBR® Premix Ex Taq™ II, 0.4µl Rox, 7.6µl nuclease-free water, 0.5µl forward primer, 0.5µl reverse primers. The following program was run on the ABI StepOne Plus real-time PCR system (Applied Biosystems Company, USA): One cycle of 95°C for the 30s, 40 cycles of 95°C for 5s, 60°C for 30s, 70°C for 15s. Real-time PCR was carried out in optical 8-cap strips (MicroAmp® Applied Biosystems, Singapore) and the β-actin as housekeeping gene was used for normalizing the amplification. The relative amounts of PCR product were calculated using the $2^{-ΔΔCt}$ method. The quality of graphs, melting curves and quantitative analyses of the data were performed using StepOne Software Version 2.2.2 (Applied Biosystems, USA).

2.6. Cell Culture

J774 cell line was grown in DMEM supplemented with 2mM glutamine, 25 mM Hepes, 100U/ml penicillin, 100mg/ml streptomycin, 10% heat-inactivated fetal bovine serum and 1.2% Na-pyruvate. Cells were plated in 24-well culture plates at a density of $2.5 \times 10^5$ cells/ml or in 60mm diameter culture dishes ($3 \times 10^6$ cells/3ml/dish) and allowed to adhere at 37°C in 5% CO2 / 95% O2 for 4h. Immediately before the experiments, the culture medium was replaced by fresh medium without fetal bovine serum and cells were stimulated as described.

2.7. Activation of COX-1

J774 cell was seeded at $2.5 \times 10^5$ cells in 1000µL per well of a 24-well plate and cultured in complete growth medium as described above. Four hours after seeding, cells were then treated with LPS-EB (1µg/ml) for 24h, after that the supernatant was replaced with fresh medium containing AA (Cayman, USA) (15µM) in the absence or presence of G2013 (5, 50 and 500mMol/ml) and incubated for a further period of 30min at 37°C in 5% CO2. After this period, the supernatant was used for the measurement of PGE2.

2.8. Activation of COX-2

In other experiment cells treated with LPS-EB (1µg/ml), to induce Cox-2, in absence or presence of G2013 (5, 50 and 500mMol/ml) and incubated for 24h at 37°C in 5% CO2. After 24h, the supernatant was used for the measurement of PGE2.

2.9. Measurement of PGE2

The measurement of PGE2 was performed by ELISA Kit (Prostaglandin E2 ELISA Kit- Monoclonal, Cayman, USA) on cell supernatants according to the manufacturer’s guidelines. A standard curve of PGE2 was prepared in parallel to the samples.

2.10. Statistical Analysis

The statistical analysis was performed using GraphPad Prism Software Version 6.02 (GraphPad Software, San Di-
3. RESULTS

3.1. Effect of G2013 on mRNA Expression of Cox-1

Our data demonstrated that the mRNA expression of Cox-1 in PBMCs of healthy donors was increased by 63% ± 17 and decreased by 149% ± 18 by G2013 at a low dose + LPS and high dose + LPS respectively, in comparison to LPS group (309% ± 14) (Fig. 2). The statistical analyses revealed that the difference between G2013 at low and high dose + LPS and control-treated with LPS (1µg/ml) were significant (p < 0.05), (p < 0.0001) respectively.

3.2. Effect of G2013 on mRNA Expression of Cox-2

Our results indicated that the mRNA expression of Cox-2 in PBMCs of healthy donors was decreased 80% ± 12 and 104% ± 15 by G2013 at a low dose + LPS and high dose + LPS respectively, in comparison to LPS group (325% ± 23) (Fig. 3). The statistical analyses revealed that the difference between G2013 at low and high dose + LPS and control-treated with LPS (1µg/ml) were significant (p < 0.05), (p < 0.0001) respectively.

3.3. Effect of G2013 on Measurement of PGE2

Our finding showed that the level of PGE2 in culture supernatants of J774 cell line was increased 29.3 ± 0.2, 58.3 ± 0.3, 62.9 ± 0.2 and 51.4 ± 0.2 by vehicle (NaOH 27mM) and G2013 (5, 50 and 500mMol/ml) respectively, in comparison to control group (3.1 ± 0.1pg/ml) (Fig. 4, left panel). The statistical analyses revealed that the difference between vehicle, G2013 (5, 50 and 500mMol/ml) and control group were significant (p < 0.0001), (p < 0.0001), (p < 0.0001), (p < 0.0001) respectively. The results also illustrated that the level of PGE2 in culture supernatants of J774 cell line was decreased 46.6 ± 0.4, 55.1 ± 0.3 and 46.0 ± 0.3 by LPS + G2013 (5, 50 and 500mMol/ml) respectively, in comparison to LPS group (144.3 ± 0.7pg/ml) (Fig. 4, middle panel). The statistical analyses revealed that the difference between LPS + G2013 (5, 50 and 500mMol/ml) and LPS group were significant (p < 0.0001). Our results indicated that the level of PGE2 in culture supernatants of J774 cell line was decreased 113.3 ± 0.2, 52.3 ± 0.6 and 58.5 ± 0.3 by AA + G2013 (5, 0.1 and 0.01mg/ml) respectively, in comparison to AA group (144.8 ± 0.6pg/ml) (Fig. 4, right panel). The statistical analyses revealed that the difference between AA + G2013 (1, 0.1 and 0.01mg/ml) and AA group were significant (p < 0.0001).

4. DISCUSSION

The small molecule of α-L-guluronic acid (G2013) [19] is a novel medicine which can be classified as an NSAID given its immunomodulatory properties. The therapeutic effects of this drug with the highest tolerability, safety, and efficacy have been proven in experimental autoimmune response. Repeated measures one-way analysis of variance (ANOVA) with Tukey’s multiple comparisons post hoc tests were utilized to analyze the differences in gene expression and activity of Cox-1/Cox-2 or a Dunnett’s test when one treatment group served as control. The results were expressed as the mean value ± standard error of the mean (SEM). A p-value < 0.05 was considered to be a statistically significant difference.
encephalomyelitis (EAE) as well as in vitro and in vivo experiments [21, 23, 27].

Afraei et al. analyzed the model of EAE and showed that the inflammation criteria including the number of inflammatory cells and plaques, and demyelination in G2013-treated mice were less than the control group. In addition, serum nitric oxide (NO) levels were significantly lower in G2013-treated mice than the control group. The results of this study indicated that treatment of mice with G2013 has a positive effect on EAE, through decreasing prevalence, severity, and progression of the disease [23]. Mirshafiey et al. showed the safe effect of G2013 on multiple factors in oxidative stress. Oxidative and nitrosative stress play an important role in aging and age-related diseases such as diabetes, inflammatory reactions, cardiovascular disease, and neurodegenerative and neuropsychiatric disorders. In this study, the expression of antioxidant enzymes such as superoxide dismutase 2 (SOD2), glutathione peroxidase (GPX1), glutathione S-transferase (GST), catalase (CAT) was increased in G2013-treated rats in comparison with the control group. In addition, the safety impact of G2013 on protein oxidation marker (PO), antioxidant capacity (TAC), and inducible nitric oxide synthase (iNOS) as a free radical-producing enzyme was confirmed, but the serum level of malondialdehyde (MDA) was significantly increased in the G2013 group compared to the control group. In addition, other aspects of the study revealed the safe effect of G2013 on cortisol steroid hormone; and there was no weight loss in the treatment group compared to rats in the control group three months after administration of the drug to 11 rats [24]. Taeb et al. showed that as a novel NSAID with immunomodulatory properties, G2013 can modify the expression of SOD2, GPX1, CAT, GST, iNOS, and myeloperoxidase (MPO) genes up to normal gene expression and may reduce the pathologic process of aging and age-related inflammatory diseases [25].

A study found that G2013 can regulate TLR4 signaling pathway without changing the expression of miRNA-146a (an anti-inflammatory agent) during inflammation through reducing the downstream signaling molecules, or interleukin-1 receptor-associated kinase (IRAK1) and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) [28]. The results of a study showed 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 [29]. In another study performed on BALB/c mice, acute and chronic toxicity had no significant clinical or histopathological impact, and evaluation of hematological and biochemical indices did not show any sign of undesirable systemic effect induced by G2013. In addition, the results showed that oral intake of G2013 at a high level of 1250mg/kg (the highest dose tested) has no adverse effect on animals. Hence, appropriate levels of this agent can be considered relatively safe for human [20]. At present, this anti-inflammatory drug is in the 1st and 2nd phases of clinical trials on ankylosing spondylitis with registration number IRCT2016091813739 and in the 1st and 2nd phases of clinical trials on rheumatoid arthritis with registration number IRCT2016092813739; the results of which will be released soon.

Since several studies indicate an important role for both COX isoforms in inflammatory and autoimmune diseases, we aimed to investigate the anti-inflammatory and immunomodulatory effects of G2013 on gene expression and activity of COX-1/COX-2 enzymes in order to introduce a novel NSAID for treating inflammatory diseases. The results showed that the low and high doses of this drug (2.5mMol/ml and 12.5mMol/ml) can significantly reduce the gene expression of COX-1/COX-2 as compared to the control treated with LPS (Fig. 2 and 3). It should be noted that LPS as an activator is able to stimulate COX-2 gene, however, this agent can also stimulate its isoenzyme COX-1 gene in the biological processes [30]. In addition, the results revealed that G2013 (5, 50, and 500mMol/ml) can significantly reduce the enzymatic activities of COX-1/COX-2 as compared to the control treated with LPS (Fig. 4, middle panel) and AA (Fig. 4, right panel). In this figure (Fig. 4, left panel) the cause of increasing level of PGE$_2$ by only G2013 is due to the effect of its solvent (NaOH 27mM) since this fact could be seen in vehicle graph in this panel.

**Fig. (4).** The effects of G2013 on quantity of PGE$_2$ in culture supernatants of J774 cell line. Left panel: The groups were unstimulated control (US), vehicle (Veh) (NaOH 27mM) and G2013 concentrations from 5-500mMol/ml. $^{****}p < 0.0001$ vs. US control group. Middle panel: Cells were control-treated with LPS (1μg/ml) and G2013 concentrations from 5-500mMol/ml. $^{****}p < 0.0001$ vs. LPS-treated group. Right panel: Cells were control-treated with AA (15μM) and G2013 concentrations from 5-500mMol/ml. $^{****}p < 0.0001$ vs. AA-treated group. Data points are given as mean ± SEM. Experiment were carried out in triplicate for each treatment group.
CONCLUSION

Many of current researchers have conducted to improve inflammation and autoimmune diseases by considering COX enzymes as a pharmaceutical target. These investigations have also tried to control the incidence and progression of inflammatory and autoimmune diseases by using NSAIDs with low toxicity and side effects. The present study revealed that G2013, as a novel NSAID with the immunomodulatory property, is able to reduce the gene expression and activity of COX-1/COX-2 enzymes. According to the findings, this drug might be categorized and introduced as a novel NSAID for the treatment of inflammatory diseases.

CURRENT AND FUTURE DEVELOPMENTS

At present, the α-L-guluronic acid (G2013) has been run in two separate clinical trials on Iranian ankylosing spondylitis and rheumatoid arthritis patients and their results under two different manuscripts are being submitted to the scientific journals. Moreover, we are running this novel drug in Multiple Sclerosis (MS) patients under another clinical trial based on getting the positive response in an experimental model of MS [23]. The future development of this drug is to run the international multicenter phase III clinical trial of α-L-guluronic acid in rheumatoid arthritis patient for introducing this agent as a novel drug for treatment of autoimmune and inflammatory diseases.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This research was approved by Ethics Committee of Rafsanjan University of Medical Sciences with registered No. IR.RUMS.REC.22.

HUMAN AND ANIMAL RIGHTS

No animals were used in the study. The study was conducted under guidelines established by Helsinki manifest and it’s later amendments or comparable ethical standards.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

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

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