Inhibitory effect of G2013 molecule as a novel immunomodulatory agent, on miR-155 gene expression in HEK-Blue hTLR4 cell line

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Abstract
Lack of regulation of microRNAs (miRNAs) expression has been observed in some autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus. G2013, as a novel non-steroidal anti-inflammatory drug (NSAID) with the immunomodulatory property, has been shown the positive effects in multiple sclerosis and anti-ageing experimental models. This research aimed to study the inhibitory effect of G2013 on miR-155 gene expression using HEK-Blue hTLR4 and Null2 cell lines. Total RNA was extracted from the treated and control cells. cDNA was made for miRNA and expression levels of miR-155 were detected by quantitative Real-time PCR using a specific primer together with U6 as the internal reference gene. A non-significant reduction was observed in the gene expression level of miR-155 in the HEK-Blue hTLR4 and Null2 cell lines under the influence of a low dose of G2013. In contrast, adding lipopolysaccharide (LPS) to the mentioned cells led to a significant increase in miR-155 expression, whereas addition of LPS four hours after exposing the cells with G2013 could not increase the expression level of this miRNA ($P < 0.05$). Collectively, this research showed that G2013, as a novel NSAID with immunomodulatory property is able to significantly decrease the gene expression of miR-155 following stimulation by LPS.

Keywords
G2013, HEK-Blue hTLR4, LPS, microRNA, miR-155, non-steroidal anti-inflammatory drug (NSAID), TLR4

Introduction
MicroRNAs (miRNAs) are endogenous non-coding RNAs with an approximate length of 22 nucleotides.1 They are the best-classified group among the well-known small RNAs in terms of number, diversity, expression, and performance.2 These small non-coding RNAs regulate gene expression at the post-transcriptional level. The regulation takes place through preventing the translation of target mRNA or through its degradation.3 miRNAs exist in a wide range of species ranging from worms to humans. Approximately 1500 miRNAs have been discovered in humans.4 Genes of miRNAs from about 3% of the entire human genome and their total number may reach the thousands.5 Using bioinformatics and molecular cloning methods, thousands of miRNAs

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have been identified in plants, animals, and viruses. miRNAs play an important role in biological processes such as cell development, proliferation, differentiation, carcinogenesis, metabolism, angiogenesis, and inflammation. Lack of regulation of miRNAs expression and function is associated with various human diseases, including cancer, heart valve defects, neurodegeneration, and autoimmunity. miRNAs play an important role in the regulation of immunological functions including acquired and innate immune responses, development and differentiation of immune cells, and prevention of autoimmunity.

The miR-155 is a miRNA, encoded in human by MIR-155 host gene or MIR155HG, and contributes to various physiological and pathological processes. The AICDA, ETS1, JARID2 (regulatory proteins for myelopoiesis and leukemia), and MyD88, SOCS1, SHIP1 (effective in inflammation), and CEBPβ, PCCD4, ZNF652 (known as tumor suppressors), can be mentioned as the targets of miR-155. The miR-155 is expressed in a variety of immune cell types, comprising B cells, T cells, macrophages, dendritic cells, and stem cells. The miR-155 is involved in autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). In an investigation, in the field of RA, it has been found that the basic miRNAs (miR-155 and miR-146a) lead to the function failure of peripheral blood mononuclear cells (PBMCs) and synovial tissue in RA patients. On the other hand, the exact function of miRNA in mouse models of lupus is unknown. However, the changes in miRNA expression in PBMCs and samples of kidney have been reported in SLE patients, miR-155 and miR-146a are the most crucial miRNAs which play essential roles in the immune system function, so that the expression of miR-155 in the urine of patients with SLE disease had a positive correlation with the severity of disease, while the expression of miR-146a was in contrast with TNF-α level in urine samples.

Toll-like receptors (TLRs) are a family of pattern recognition receptors which are classified based on homology of the cytoplasmic domain with that of the interleukin-1 receptor (IL-1R) family, which is known as the Toll/IL1-R (TIR) domain. It is estimated that mammals have 13–15 types of TLR. Thirteen of these receptors, including TLR1 to TLR13, have been identified in humans and mice. These receptors are expressed in immune cells such as macrophages, dendritic cells, neutrophils, and monocytes. Most TLRs are located on the cell membrane and bind to pathogen-associated molecular patterns (PAMPs). TLR4 is the main recognition receptor of lipopolysaccharide (LPS) (also known as endotoxin), a Gram-negative bacterial cell wall component. The recognition process is increased by LPS-binding protein (LBP) that carries LPS to the CD14 molecule, where it binds to the MD2-TLR4 complex. MD2 is a molecule which binds to the extracellular part of TLR4 and increases response to LPS. Activation of TLR4 leads to stimulation of MyD88-dependent and MyD88-independent pathways. These pathways regulate the balance between cell survival and inflammation. Some miRNAs are induced by TLR signaling and negatively regulate the signaling process. The expression of miR-155-5p can greatly improve following stimulation of TLR agonist of macrophages and dendritic cells. It has also been found that miR-155 expression is activated in mouse macrophages treated with LPS through an NF-κB mediated mechanism. The activated miR-155-5p inhibits negative regulators of inflammation, including SHIP1 and SOCS1. This inhibition improves cell survival, growth, and migration and anti-pathogenic responses.

G2013 is a small molecule with uronic acid structure patented (DE-10247073), as a novel non-steroidal anti-inflammatory drug (NSAID) with the immunomodulatory property was prepared in immunology department of Tehran University of Medical Sciences, which has been tested in several experimental models. It should be noted that NSAIDs play an important role in the management of inflammatory diseases. During recent years, researchers have tried to identify safer and more effective types of anti-inflammatory and immunomodulatory drugs.

Therefore, regarding the role of miR-155 in inflammation and autoimmune diseases, the effect of G2013 on the expression level of miR-155 in HEK-293 transfected cells (HEK-Blue hTLR4 and Null2) was investigated in connection with LPS effects.

Materials and methods

Cell line and cell culture

Engineered human embryonic kidney (HEK) cell line HEK-Blue hTLR4 and Null2 (the gift from M. Yousefi) were cultured in complete growth medium
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(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 the presence of cell linespecific 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, and for Null2 cells 100 µg/mL Zeocin only (InvivoGen, USA). The HEK-Blue hTLR4 reporter cell line was stably transfected with human TLR4, MD2/CD14 co-receptor, and the secreted embryonic alkaline phosphatase (SEAP) under the control of an NFκB-responsive promoter. Null2 cells served as the parental control cell line and were stably transfected with the NF-κB-responsive, SEAP reporter only. Cultures were grown in 25 cm² flasks at 37°C and 5% carbon dioxide (CO₂). Cells were subcultured when they were 60–80% confluent. The media were changed two to three times a week.

Treatment of cell with G2013 and endotoxin

HEK-Blue hTLR4 and Null2 Cells were seeded at 5 × 10⁵ 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 (2.5 mMol/well), G2013 high dose (7.5 mMol/well), LPS-EB (LPS from E. coli O111:B4) (1 µg/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.

RNA extraction and reverse transcription

RNA was extracted from control and treated cells using Hybrid-R™ Mini kit (GeneAll, Republic of Korea) according to the manufacturer’s guidelines. The extracted RNA quality was identified by electrophoresis and the GelRed™ (Biotium, USA) contained agarose gel and measured absorption on A260/280 nm by NanoDrop® ND1000 spectrophotometer (Isogen Life Science, The Netherlands). cDNA was synthesized using a MystiCq® microRNA cDNA synthesis Mix (Sigma-Aldrich). cDNA synthesis reactions contained 6 µL cells RNA, 2 µL poly (A) Tailing Buffer (5×), 1 µL poly (A) polymerase, 1 µL nuclease-free water, 9 µL MystiCq microRNA cDNA Reaction Mix, 1 µL ReadyScript Reverse Transcriptase. The 20 µL of reactions were incubated for 60 min at 37°C, 5 min at 70°C, 20 min at 42°C, 5 min at 85°C, and held at 4°C.

Table 1. Primers and sequences of hsa-miR-155-5p and RNU6-1.

<table>
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<th>Primers</th>
<th>Sequence</th>
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<tr>
<td>hsa-miR-155-5p</td>
<td>F: 5’-UUAAUGCUAAUCGUGAUAGGGGU-3’</td>
</tr>
<tr>
<td>RNU6-1</td>
<td>F: 5’-GUGCUCGCUUCGGCAGCAC</td>
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Quantitative real-time PCR

Quantitative real-time PCR was performed using SYBR® Premix Ex Taq™ II (Dalian, Takara Co., Ltd) with specific primer (Sigma-Aldrich) (Table 1).

Twenty microliters of real-time PCR reactions included 0.5 µL genomic template DNA, 10 µL SYBR® Premix Ex Taq™ II, 0.4 µL Rox, 8.1 µL nuclease-free water, 0.5 µL forward primer (10 pM), 0.5 µL universal primer (10 pM) (both by Sigma-Aldrich). The following program was run on the ABI StepOne Plus real-time PCR system (Applied Biosystems Company, USA): one cycle of 95°C for 30 s, 40 cycles of 95°C for 5 s, 60°C for 30 s, and 70°C for 15 s. Real-time PCR was carried out in optical 8-cap strips (MicroAmp® Applied Biosystems, Singapore) and the U6 small nuclear RNA as housekeeping gene was used for normalizing the amplification. The relative amounts of PCR product were calculated using the 2⁻ΔΔCt method (Ct refers to the threshold value). The quality of graphs, melting curves, and quantitative analyses of the data were performed using StepOne Software Version 2.2.2 (Applied Biosystems, USA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism Software Version 5.00 (GraphPad Software, San Diego, CA, USA; www.graphpad.com). A one-way analysis of variance (ANOVA) with Newman-keuls multiple comparison post hoc test was utilized to analyze differences in expression of miR-155 or a Dunnett’s test when one treatment group served as control. The results are expressed as the mean value ± standard error of the mean (SEM). A P value <0.05 was considered to be a statistically significant difference. **P <0.01, ***P <0.001, and ###P <0.001.
Results

Effects of G2013 on miR-155 expression in HEK-Blue hTLR4 cell line

Our results showed that the expression of miR-155 in HEK-Blue hTLR4 cell lines decreased by 22.7% ± 15.6 and increased by 20% ± 15.6 by G2013 at a low dose and high dose, respectively, in comparison to the control group (100%) (Figure 1). The statistical analyses revealed that the difference between control group and other groups were not significant ($P = 0.35$). The results also demonstrated that the expression of miR-155 was decreased 265% ± 35.6 and 185% ± 35.6 by G2013 at a low dose + LPS and high dose + LPS, respectively, in comparison to the LPS group (337% ± 14.3) (Figure 2). Statistical analyses revealed that the difference between G2013 low dose + LPS and control-treated with LPS (1 µg/mL) was significant ($P < 0.001$). Moreover, the difference between G2013 high dose + LPS and LPS was also significant ($P < 0.001$).

Effects of G2013 on miR-155 expression in HEK-Blue Null2 cell line

Our complementary data showed that the expression of miR-155 in the HEK-Blue Null2 cell lines (cell line without TLR4 and MD2/CD14 receptors) decreased by 42.7% ± 22 and increased by 31.1% ± 22 by G2013 at a low dose and high dose, respectively, in comparison to the control group (100%) (Figure 3). The statistical analyses revealed that the differences between the control group and other groups were not significant ($P = 0.24$). The results also demonstrated that the expression of miR-155 decreased by 63.6% ± 14.4 and increased by 9.7% ± 22 by G2013 at a low dose and high dose, respectively, in comparison to the control group (100%).

Figure 1. The effect of G2013 on miR-155 expression in HEK-Blue hTLR4 cell line. The groups were untreated control and G2013 treated with two doses, low (2.5 mMol/well) and high (7.5 mMol/well). Data points are given as mean ± SEM. Experiments were carried out in triplicate for each treatment group.

Figure 2. The effect of G2013 along with LPS on miR-155 expression in HEK-Blue hTLR4 cell line. The groups were untreated control, LPS-treated (1 µg/mL) and G2013 with two doses, low (2.5 mMol/well) and high (7.5 mMol/well). Data points are given as mean ± SEM. Experiments were carried out in triplicate for each treatment group. ***$P < 0.001$ vs. LPS-treated group and ###$P < 0.001$ vs. untreated control group.

Figure 3. The effect of G2013 on miR-155 expression in HEK-Blue Null2 cell line. The groups were untreated control and G2013 treated with two doses, low (2.5 mMol/well) and high (7.5 mMol/well). Data points are given as mean ± SEM. Experiments were carried out in triplicate for each treatment group.
± 14.4 by G2013 at a low dose and high dose, respectively, in comparison to LPS group (111% ± 10.1) (Figure 4). The statistical analyses revealed that the difference between G2013 low dose + LPS and control-treated with LPS (1 µg/mL) was significant (P < 0.01), while the difference between G2013 high dose + LPS and LPS was not significant (P = 0.83).

Discussion

miRNAs are highly conserved molecules of the regulated intercellular RNAs which control gene expression at the post-transcriptional level. These molecules can often partially bind themselves to the 3'-untranslated region of the target messenger RNA (mRNA) to suppress translation of the target mRNA or to induce its degradation. Approximately more than 1000 miRNAs have so far been identified, which regulate about 30% of human gene expressions. Numerous reports suggest that miRNAs play an important role in regulating immune responses and development of autoimmune disorders. Many studies have confirmed the involvement of miR-155 in differentiation and activation of innate and adaptive immune systems. The miR-155 acts more effectively in differentiating Th1 and Th2 cells from TCD4+ cells. Moreover, miR-155 plays a role in myeloid cells differentiation as well as B cells development. Recently, it was demonstrated that miR-155 plays an important role in autoimmune diseases such as RA, SLE, and possibly multiple sclerosis (MS). Increased expression of miR-155 in PBMCs and synovial fibroblasts of patients with RA has been reported. This increased expression was also observed in urine and serum samples of patients with SLE. Recent studies have shown that miR-155 and miR-326 expression occurs in TCD4+ cells in mice model of experimental autoimmune encephalomyelitis (EAE). These miRNAs are responsible for regulating the severity of diseases caused by Th17. Expression of miR-155 is induced by TLR signaling. The miR-155 via targeting MyD88, SOCS1, SHIP1, TAB2, and IKKε negative regulates TLR signaling. Since microbial lipopolysaccharide (an agonist of TLR4) activates a chain of events which stimulates the transcription factors NF-κB and AP-1, it was assumed that activation of endotoxin MIR155HG might be mediated by these transcription factors. A relationship between miR-155 and innate immune response was suggested by Tali and O’Connell’s studies, which showed an upregulated expression after stimulation with LPS (via TLR4) and lipoprotein (via TLR2) in monocytes or macrophages and spleen cells in the mice inoculated by Salmonella enteritidis-derived LPS. O’Connell et al. proved that LPS induces a strong but transient expression of miR-155 in bone marrow cells of mice, suggesting that this would likely lead to granulocyte/monocyte expansion. Further research has shown that the increased expression of miR-155 via targeting SOCS1 and SHIP1 in PBMCs and synovial fluid of patients with RA would increase production of inflammatory cytokines. According to the research conducted on G2013, this molecule has shown the significant positive effects on the experimental model of MS. Advantages of G2013 over other NSAIDs include its low molecular weight and natural base, immunomodulatory and anti-ageing effects with its high safety property. Based on the chemical structure of this molecule, we predict that it might be attached to the pattern recognition receptors (PRR) associated molecules, probably (TLRs, CD14, and/or Mannose receptors) in endothelial, monocytes, macrophages, and dendritic cells. Since miR-155 plays a role in the molecular mechanisms of autoimmune diseases, we made it our purpose in this study to examine...
the effect of G2013 as an immunomodulatory drug on the expression of miR-155 using HEK-Blue hTLR4 and Null2 cell lines. In this study, following the effect of G2013 in low and high doses on the above mentioned cell lines, it was found that at low dose this drug can significantly decrease the expression of the miR-155 gene compared with the LPS-treated group (Figures 2 and 4), which could be an important pharmaceutical target for reducing the inflammatory reactions. Collectively, in recent years, many researchers have considered miR-155 as a therapeutic target in order to suppress autoimmune diseases. This study showed that G2013 as a novel immunomodulatory agent can significantly reduce the gene expression of miR-155 following stimulation by LPS.

Declaration of conflicting interests
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References
21. Pauley KM, Satoh M, Chan AL et al. (2008) Upregulated miR-146a expression in peripheral blood


