Original article

Pharmacological effects of β-ν-mannuronic acid (M2000) on miR-146a, IRAK1, TRAF6 and NF-κB gene expression, as target molecules in inflammatory reactions

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ABSTRACT

Background: Impaired expression and function of microRNAs (miRNAs) are involved in the pathogenesis of many autoimmune and inflammatory diseases. Moreover, there is a close relationship between TLRs and miRNAs and impairment in regulating their expression which can play a vital role in the immunopathogenesis of many inflammatory reactions. This research aimed to study the pharmacological effects of M2000 (β-ν-mannuronic acid) on the expression of miR-146a and its two target molecules (IRAK1 and TRAF6), and the transcription factor NF-κB in the HEK-Blue hTLR2 cell line.

Methods: The cytotoxicity of M2000 was assessed by the MTT assay, and the qRT-PCR technique was employed in the presence and absence of M2000 treatment to measure gene-expression levels of miR-146a, IRAK1, TRAF6, and NF-κB.

Results: MTT assay indicated that M2000 (before the concentration of 500 μg/ml) had no cytotoxic effect on HEK-Blue hTLR2 cells. Our results showed that M2000 at low and high doses (5 and 25 μg/well) could significantly reduce gene expression levels of miR-146a (p < 0.01). Furthermore, it was found that this medication at two different doses could considerably decrease IRAK1 and TRAF6 gene expression (p < 0.01). Moreover, this study revealed that expression level of NF-κB also significantly declined at these two doses (p < 0.01).

Conclusions: This study for the first time shows that M2000 as a novel NSAID with immunosuppressive properties is able to modify TLR signaling through suppressing the adaptor molecules IRAK1 and TRAF6, the transcription factor NF-κB and miR-146a as a new therapeutic approach.

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Introduction

MicroRNAs (miRNAs) are the new class of single-stranded, non-coding RNAs in length of about 18–25 nucleotides and their role has been shown so far in regulating various biological processes such as growth, cell cycle, development, differentiation, metabolism, apoptosis, angiogenesis, and inflammation. The miRNAs exert their effects through regulation of gene expression at the post-transcriptional level so that they prevent the production of protein through destruction or inhibition of translation of the target mRNA. The miRNAs are found in a wide range of different species, from worms to humans. Approximately 1500 miRNAs have been detected in humans. The miRNA genes make up about 3% of the total human genome which may increase to thousands of
these genes [5]. Dysregulation of miRNAs expression and function is associated with different human diseases including cancer, heart valve defects, neurodegeneration, and autoimmune [6].

The miR-146a is a miRNA which plays an important role in hematopoiesis, lymphopoiesis, cancer, and inflammation and its encoding gene is located on chromosome 5 in locus 5q33.3 in humans [7]. The miRNAs were discovered during investigations on miRNAs involved in innate immunity in response to bacterial infection. The study of human lung alveolar epithelial cells led to the identification of targets of miR-146a which controls innate immunity. In addition, it was found that the expression of this miRNA is associated with nuclear factor-κB (NF-κB) gene activity [8]. Among target molecules of miR-146a, one can point out interleukin–1 receptor-associated kinase 1 (IRAK1), tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), chemokine (C-C motif) ligand 8 (CCL-8), fas-associated protein with death domain (FADD), interferon regulatory factor 5 (IRF-5), and signal transducer and activator of transcription 1 (STAT-1) [9–12]. The miR-146a is involved in autoimmune diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and psoriasis (Ps). It was shown in a study on RA that essential miRNAs, including miR-146a and miR-155, cause failure in peripheral blood mononuclear cells (PBMCs) and synovial tissue of RA patients [13]. On the other hand, although the exact function of miRNAs in mouse models of lupus is unknown, however, the expression of miRNAs changes in PBMCs and kidney samples in patients with SLE. A study showed that the expression of miR-146a and miR-155 reduces in the serum of SLE patients [14]. It was shown in another study on PS that the expression of miR-146a increases significantly in these patients [15].

Toll-like receptors (TLRs) are a family of pattern recognition receptors which are classified with interleukin receptor-1 (IL-1R) family based on homology in a cytoplasmic domain known as Toll/IL-1R domain [16]. It is estimated that 13–15 TLR types exist in mammals [17]. Thirteen of these receptors, including TLR1 to TLR13, have been identified in humans and mice [18,19]. These receptors are expressed in immune cells such as macrophages, dendritic cells, neutrophils, and monocyes. There are more TLRs on the cell membrane which are connected to pathogen-associated molecular patterns (PAMPs) [20]. PAMPs structure, including lipopolysaccharides (LPS), lipoteichoic acid (LTA) and peptidoglycan are conserved on various microorganisms [18]. TLR2 is able to detect a variety of microbial products, such as lipopeptides, lipopolysaccharides, LTA, β-glucan through heterodimers formation with others TLRs [21]. Activation of TLR2 leads to stimulation of both myeloid differentiation factor 88 (MyD88)-dependent and MyD88-independent pathways [20]. These pathways regulate the balance between cell survival and inflammation. Some miRNAs are induced by TLR signaling and negatively regulate signaling [22]. TLR signaling induces the expression of miR-146, miR-155, miR-21, and some other miRNAs [23]. The expression of miR-146a in monocytes increases in response to activation of signaling pathways from TLR2, TLR4, and TLR5 receptors or from TNFα and IL-1β receptors [24]. There are several NF-κB transcription factor binding sites in the promoter of miR-146a gene, and miR-146a gene expression by LPS, IL-1β, and TNF-α is dependent on NF-κB [8]. IRAK1 and TRAF6 are two key adapter molecules downstream to TLRs and IL-1 receptors (TIRs) signaling pathway and play a central role in signal transduction events [25,26].

Evidence has shown that induction of miR-146a in innate immune cells can subsequently reduce IRAK1 and TRAF6 adaptor molecules which in turn can inhibit the activation of the transcription factor NF-κB and hence suppression of NF-κB target genes expressions such as IL-6, IL-1β, and TNFα [24].

**Fig. 1.** The chemical structure of M2000 (β-0-mannuronic acid) patented (DE-102011130384.4).

M2000 (β-0-mannuronic acid) as a novel designed non-steroidal anti-inflammatory drug (NSAID) plays an important role in the management of inflammatory diseases (Fig. 1) [27]. Alginates consist of (1_4) linked β-0-mannuronic acid (M-block) and α-1-guluronic acid (G-block) residues of widely varying in composition and sequence [28,29]. Alginates are abundant in nature since they are seen in both capsular polysaccharides in some bacteria and as a structural element in marine brown algae. To date, researchers have tried to identify the safer and more effective types of anti-inflammatory drugs. M2000 molecule is an agent with the lowest molecular weight and has less toxicity compared with other NSAIDs [30].

The present study aimed to investigate the anti-inflammatory and immunosuppressive effects of M2000 in both low and high dose on the expression of miR-146a and its two target molecules (IRAK1, TRAF6) and the transcription factor NF-kB in the HEK293 transfected cells (HEK-Blue hTLR2).

**Material and methods**

**Cell line and cell culture**

Engineered human embryonic kidney (HEK) cell line HEK-Blue hTLR2 (the gift of M.Yousefi) was cultured in complete growth medium (RPMI 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 (Bioc, Life Technologies, USA) and 100 μg/ml NormocinTM (InvivoGen, USA)) in presence of cell line-specific selection antibiotics. The selected antibiotics for HEK-Blue hTLR2Cells contained 100 μg/ml ZeocinTM, 200 μg/ml Hygromycin B gold and 30 μg/ml Blasticidin. The HEK-Blue-hTLR2 cells are HEK 293 cells stably expressing human TLR2 (hTLR2) receptor and the needed accessory proteins to TLR2, MD-2, and CD14. These accessory proteins interact with LTA at the TLR2 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 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.

**MTT cytotoxicity assay**

Cell viability and cytotoxicity studies of M2000 were performed by MTT assay. The cell counting was conducted using Trypan blue
and HEK-Blue hTLR2Cells were then seeded (1 × 10^4 cells per well) in 96-well plates. 24 h after seeding, two-fold dilutions of β-o-Mannuronic RNA microplate primer, manufacturer’s agarose cDNA (Netherlands). Following F, Table Applied Biosystems, 96-well reaction buffer (10 μM), 2 μl Nuclease-free water, 2 μl RT 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 DNA, 10 μl SYBR® Premix Ex Taq™ II, 0.4 μl Rox, 7.6 μl Nuclease-free water, 0.5 μl forward primer, 0.5 μl 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, 72 °C for 34s. 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).

cDNA synthesis and qRT-PCR for measuring IRAK1, TRAF6, NF-κB

Total RNA from control and treated cells was reverse transcribed using HyperScript™ First-strand Synthesis Kit (GeneAll, Republic of 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 μM), 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.).

RNA extraction

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 on the GelRed™ (Biotium, USA) contained agarose gel and measured absorption on A260/280 nm by NanoDrop™ ND1000 spectrophotometer (Isogen Life Science, Netherlands).

cDNA synthesis and qRT-PCR for measuring miR-146a

After extraction of total RNA from control and treated cells, cDNA was synthesized using a MystiCq® microRNA cDNA synthesis Mix (Sigma-Aldrich). The cDNA synthesis reactions contained 6 μl total RNA, 2 μl Poly (A) Tailing Buffer (5 ×), 1 μl Poly (A) Polymerase, 1 μl Nuclease-free water, 9 μl Mystiq 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. Real-time PCR was performed using SYBR® Premix Ex Taq™ II (Dalian, Takara Co., Ltd.), produced cDNA and specific miRNA 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, 0.5 μl universal primers (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 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 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. The quality of graphs, melting curves and quantitative analyses of the data were performed using StepOne Software Version 2.2.2 (Applied Biosystems, USA).

Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRAK1</td>
<td>F: 5′-TGAAGACGCTGAAAGAGAC-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-CTGACCATGGTACAGAG-3′</td>
</tr>
<tr>
<td>TRAF6</td>
<td>F: 5′-TGGCGTACATTTTCTGAGTG-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-ATCCAGAAGACATGCTACTC-3′</td>
</tr>
<tr>
<td>NF-κB</td>
<td>F: 5′-GCTACAGGGACCAGAGACGTT-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-GCTCAGCTGTATAGAGCCAC-3′</td>
</tr>
<tr>
<td>β-actin</td>
<td>F: 5′-GGCCCTTCTCCTCGGATGTC-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-GGACCTGGTCTGGCTGACGTC-3′</td>
</tr>
<tr>
<td>miR-146a</td>
<td>F: 5′-UCAGAACACCAAUAUCUAGGUU-3′</td>
</tr>
<tr>
<td>U6</td>
<td>F: 5′-GUGCUGCCGCUUGGACACAAUAG-3′</td>
</tr>
</tbody>
</table>

F, forward; R, reverse.

Statistical analysis

Statistical analysis was performed using GraphPad Prism Software Version 6.00 (GraphPad Software, San Diego California USA; www.graphpad.com). A one-way analysis of variance (ANOVA) with Tukey’s multiple comparisons post hoc tests was utilized to analyze of genes expression between treated group and untreated (control). The results are expressed as the mean value ± standard deviation (SD). A p-value < 0.05 was considered to be a statistically significant difference; *p < 0.05, **p < 0.01, ***p < 0.001.

Results

Cytotoxicity measurement of M2000

To investigate the effects of M2000 on the cell survival of HEK-Blue hTLR2 cell line, these cells were incubated in the presence or absence of various concentrations of M2000 (0–1000 μg/ml) for 24 h and then subjected to MTT cytotoxicity assay. M2000 showed inhibitory effects on the cell growth rate of HEK-Blue hTLR2 cells in a concentration-dependent manner. M2000 IC50 was 1000 μg/ml after 24 h treatment (p < 0.05) (Fig. 2).

Effect of M2000 on gene expression of miR-146a

Our results showed that expression of miR-146a in HEK-Blue hTLR2 cell lines was decreased 52.56 ± 12.2 and 64.8% ± 12.2 by M2000 at a low dose and high dose respectively, in comparison to control group (100%) (Fig. 3). The statistical analyses revealed that the difference between M2000 low dose and control group was significant (p < 0.05). Moreover, the difference between M2000 high dose and control group was also significant (p < 0.01).
Effect of M2000 on expression of target genes

Our data demonstrated that expression of IRAK1 in HEK-Blue hTLR2 cell line was decreased 24.6% ± 4.8 and 39.2% ± 4.8 by M2000 at a low dose and high dose respectively, in comparison to control group (100%) (Fig. 4). The statistical analyses revealed that the difference between M2000 low dose and control group was significant (p < 0.01). Moreover, the difference between M2000 high dose and control group was also significant (p < 0.001). The results also showed that the gene expression of TRAF6 in HEK-Blue hTLR2 cell line was decreased 54.2% ± 7.4 and 56.1% ± 7.4 by M2000 at a low dose and high dose respectively, in comparison to control group (100%) (Fig. 5). The statistical analyses revealed that the difference between M2000 low dose and control group was significant (p < 0.001). Moreover, the difference between M2000 high dose and control group was also significant (p < 0.001).

Effect of M2000 on gene expression of NF-κB

Our finding showed that the gene expression of NF-κB in HEK-Blue hTLR2 cell lines was decreased 36.9% ± 9.8 and 62.1% ± 9.8 by M2000 at a low dose and high dose respectively, in comparison to control group (100%) (Fig. 6). The statistical analyses revealed that the difference between M2000 low dose and control group was significant (p < 0.05). Moreover, the difference between M2000 high dose and control group was also significant (p < 0.01).

Discussion

The miR-146a is one of the negative post-transcription regulators in signal transduction of immune cells through TLRs (TLR2, TLR4) [31]. During the inflammation process, TLRs react to its specific ligand; and TLR signaling leads to the activation of two adapter molecules IRAK1, TRAF6; and ultimately activation of NF-κB as the main transcription factor of inflammatory cytokines [32]. In contrast, the excessive activation of NF-κB induces the...
Fig. 6. The effects of M2000 on the level of NF-κB mRNA in the HEK-Blue hTLR2 cell line. Cells were treated for 24 h with two doses, low (5 μg/well) and high (25 μg/well) of M2000, and NF-κB mRNA expression was measured by qRT-PCR using total RNA extracted from control and treated cells. Values were normalized to the β-actin content of the samples. The results were expressed as the target/reference ratio of the treated samples divided into the target/reference ratio of the untreated control sample and expressed as mean ± SD; *p < 0.05 and **p < 0.01 vs. control group.

expression of miR-146a in the innate immune cells leading to the decreased expression of the genes IRAK1, TRAF6, NF-κB [33].

Changes in gene expression of miR-146a have been reported in the pathogenesis of many autoimmune diseases including RA, SLE, PS and Sjögren’s syndrome (SS) which can affect its targets in different ways. Nakasa et al. showed that miR-146a expression in synovial tissue of patients with RA increases intensively compared with normal synovial tissue and osteoarthritis (OA) [34]. In a study on SLE, it was found that the expression level of miR-146a is negatively correlated with disease severity and the amount of interferon [35]. Another study reported the increased expression of miR-146a in SS patients [36]. The evidence also showed that the expression level of miR-146a in patients with PS can specifically increase [15].

IRAK1 and TRAF6 are two main adapter molecules between TLR2 and NF-κB signaling pathways, and they are the targets of miR-146a [9,21]. In a study, Maitra et al. demonstrated that the expression level of RORyt and IL-17A is reduced in T cells lacking the gene IRAK1, after treatment with TGF-β1 and IL-6; while the expression level of Foxp3 increases which indicates an important role of IRAK1 in regulating the balance between regulatory T cells and Th17 [37]. In pre-clinical studies of multiple sclerosis, it has been shown that mice with defective gene IRAK1 are resistant to laboratory autoimmune disease; and histological analysis of the central nervous system does not show any inflammation and/or the inflammation could be minimal which indirectly down-regulates the Th1 response in reducing the production of IFN-γ [38]. In another study, Wu et al. reported that dendritic cells of mice with defective gene TRAF-6, are deficient in terms of the performance of cytokine production and up-regulation of costimulatory molecules in response to CD40L, associated with microbial components in vitro and in vivo conditions [39].

TLRs have a role in many reactions and autoimmune and inflammatory disorders, and many studies have shown the role of TLR in the pathogenesis of disease; which are considered until now, as a therapeutic target [40]. The use of TLR antagonists for some autoimmune and inflammatory diseases has been proposed as a promising therapeutic strategy [41].

M2000 (β-o-mannuronic acid) has been tested as an anti-inflammatory and a novel immunosuppressive agent in various experimental models, such as animal models of multiple sclerosis, nephrotic syndrome, rheumatoid arthritis and immune complex glomerulonephritis [42–44]. The molecular mechanism of therapeutic efficacy of this novel patented (DE-102016113018.4) drug is based on its inhibitory effects on matrix metalloproteinase 2 activity, immune cells infiltration in inflammatory foci, reduction of the level of inflammatory cytokine IL-6, a decrease in antibody production, and induction of apoptosis using fibrosarcoma cell line [43]. Based on our research on an extensive scale, mannuronic acid is a very safe anti-inflammatory and immunosuppressive drug and also, it has the lowest molecular weight with no gastrointestinal toxicity, in comparison with conventional NSAIDs such as diclofenac. This drug without having a ulcerogenic effect on the stomach of animals revealed a potent therapeutic efficacy in disease control [45]. Now, this anti-inflammatory drug has been tested in clinical trial phase 1/2 with the registered No. IRCT2013062213739N1 in patients with rheumatoid arthritis; and its results will soon be published.

According to the chemical structure of this molecule (Fig. 1), it can be predicted that it attaches to the cell surface receptors especially TLRs available on endothelial cells, monocytes, macrophages, dendritic cells; and in this way influences cell signaling. Because recent studies have shown the important role of TLRs in autoimmune diseases, and it seems that interfere with signaling pathways of TLR is involved in many of these diseases, the aim of this study was to evaluate the anti-inflammatory and immunosuppressive effects of M2000 on the expression of miR-146a gene and its two target molecules (IRAK1, TRAF6), as well as the transcription factor NF-κB in the cell line HEK-Blue hTLR2. In this study, following the impact of the M2000 with a low dose (5 μg/well) and high dose (25 μg/well) on HEK-Blue hTLR2 cells, it was found that this drug, in two doses, can significantly reduce the expression level of miR-146a gene (Fig. 3). It was also found that two doses of this drug can reduce IRAK1 and TRAF6 genes expression levels, significantly (Figs. 4 and 5). In addition, the present study indicated that the expression level of the gene NF-κB (as the main factor of the inflammatory transcription of cytokines) is significantly reduced in both low and high doses (Fig. 6). Collectively; while considering TLRs as therapeutic target for inhibiting the autoimmune diseases and controlling the progression of autoimmune and inflammatory reactions using their antagonists, the present research designed for the first time to show that M2000, as a novel NSAID with immunosuppressive properties is able to modify TLR2 signaling through inhibition of the adapter molecules IRAK1 and TRAF6, the transcription factor NF-κB, miR-146a and finally the reduction of proinflammatory cytokines production.

References

Like Sidiropoulos, Taganov, Shotorbani, Atarod, Nahid, protein.

miR-146a modulator (50):34590

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KD, expression 9.

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Front recommendations – M, MM, MI, 2000;164(10):5277


