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The Efficacy of Two Specific Toll-Like Receptor 4 Antagonists (G2013 & M2000) In Clinical Inflammatory Disease

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Abstract:
Toll-like receptors (TLRs) are receptors of the innate immune system that detect pathogen- associated molecular patterns and endogenous "danger" molecules. Among the family, the fundamental role of Toll-like receptor 4 (TLR 4) has been underscored in the initiation of pro- inflammatory cellular signaling pathways. Therefore, the appropriate suppression of Toll-like receptor 4 signaling is vital to maintain the balance between autoimmunity and inflammatory responses to avoid detrimental effects caused by the host immune system. This paper reviewed the structure of TLRs and their essential role in inflammation, specifically Toll-like receptor 4, its signaling cascade, and its antagonists. Recently, using two new drugs, M2000 (β-D-Mannuronic acid) and G2013 (α-L-Guluronic acid), the novel Toll-like receptor 4 antagonists are proposed to control inflammatory conditions. Immunosuppressive and anti-inflammatory properties of M2000 and G2013 have been examined in invitro, pre-clinical, and clinical trial studies. Experimental and clinical studies on these drugs revealed TLR4 antagonistic properties with significant efficacy for controlling inflammatory responses and treating autoimmune diseases.

Keywords: Toll-like receptor 4, Mannuronic acid, Guluronic acid, M2000, G2013, Inflammation

TOLL-LIKE RECEPTORS

The immune system consists of two closely related systems known as the innate and adaptive immune systems. While the adaptive immune system responds to specific "non-self" antigens and generates an immunological memory, the innate immune system runs an immediate first line of defense against various invading pathogens. Cognate pattern recognition receptors (PRRs), critical mechanisms of innate immunity, act as sentinels against both invading organisms bearing PAMPs [1], and damage-associated molecular patterns (DAMPs) consist of endogenous molecules released from stressed or dying cells, including hyaluronan, heat-shock proteins (HSPs) and fibronectin that promoted inflammatory pathway [2]. Toll-like Receptors are the most famous members of PRRs [3]. They are a large group of type I transmembrane proteins, of which thirteen of them have been identified in humans and mice [4], and facilitate the recognition of PAMPs or endogenous "danger" molecules [5-7]. Each TLR seems to recognize distinct PAMPs derived from various microorganisms, such as bacteria, viruses, protozoa, and fungi. Recognition of their ligands leads to the induction of signaling events and results in acute host responses that eventually causes pathogens' death [8, 9].

Thirteen recognized TLRs could sense a wide variety of pathogen structures, including triacyl lipopeptides by TLR1 in association with TLR2, lipoteichoic acid and lipoproteins of Gram-positive

bacteria by TLR2, double- stranded RNA by TLR3, LPS of Gram-negative bacteria by TLR4, bacterial flagellin by TLR5, diacyl lipopeptides by TLR6 in association with TLR2, single-stranded RNA by TLR7 and nonmethylated CpG of bacterial DNA by TLR9 [8].

Among these identified types, humans express ten functional TLRs (TLR1 to TLR10), whereas twelve TLRs (TLR1 to TLR9 and TLR11 to TLR13) exist in mice. Human TLR10, mouse TLR12, and mouse TLR13 are the only types whose ligands have not been identified yet [9].

Cell locations divided TLRs into two groups. The first group includes TLR1, TLR2, TLR4, TLR5, and TLR6, which are located on the cell surface, and the second one comprises TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13 that are within endosomes, endoplasmic reticulum (ER), multivesicular bodies, and lysosomes [10]. This localization prevents autoimmunity and inappropriate immune responses [11, 12].

Generally, TLRs are expressed broadly in tissues and different cells, both immune and non-immune ones. Cytokines or pro-inflammatory mediators are produced after TLRs stimulation in these cells [10, 13, 14].

TLRs are glycoproteins that consist of 3 domains: a transmembrane domain, an amino-terminal ectodomain, and a cytoplasmic carboxy-terminal Toll IL1-1R homology (TIR) domain [15, 16]. The TIR domain is the part that identifies explicitly microbial components and, as a final point, triggers the activation factors that cause the production of pro-inflammatory cytokines [17]. TLR1, TLR2, TLR4, and TLR6 recruit TIR domain-containing adaptor protein (TIRAP), which serves as an adaptor between the TIR domain of TLRs and myeloid differentiation factor 88 (MyD88), while TLR5, TLR7, TLR9, and TLR11 can recruit MyD88 directly. The binding of TLR3 and TLR4 ligands results in the recruitment of Toll/IL-1R domain-containing adaptor, inducing IFN-β (TRIF). However, the recruitment of TRIF by TLR4 needs the participation of a TRIF-related adaptor molecule (TRAM) [1].

In the inflammation process, an increase in a multitude of pro-inflammatory proteins requires signal transduction processes by activated TLRs, including Nuclear Factor-κB (NF-κB) and 2 Mitogen-activated protein (MAP) kinases, p38, and Jun N-terminal kinase (JNK) [5].

Receptor-proximal proteins involved in signaling by all TLRs include the adapter MyD88, Interleukin-1 receptor- associated kinase 1 (IRAK-4, IRAK, and IRAK-2), Toll interacting protein (Tollip), TNF-receptor-associated factor 6 (TRAF-6), and transforming growth factorβ-activated kinase 1 (TAK-1) [18, 19].

TLRs family triggers inflammation by two pathways: a canonical pathway using MyD88 adaptor protein (MyD88- dependent) and a non-canonical pathway using TRIF adaptor protein (MyD88-independent). Excluding TLR3, the canonical pathway activates MAPK and NF-κB, leading to the secretion of inflammatory cytokines such as Interleukin-6 (IL-6) and Tumor Necrosis Factor α (TNF-α). On the other hand, the non-canonical pathway motivates Interferon Regulatory Factor 3 (IRF3) due to interferon production [20].

The development of cancers and inflammatory diseases, such as neuro-inflammation, autoimmune and cardiovascular diseases, may occur due to TLRs activation and excessive

activation of inflammatory pathways [21-23]. Interestingly, TLRs play anti-tumor or tumor-developing roles in different cancer cells [24]. Furthermore,

it has been suggested that unsuitable TLR recognition and signaling pathways play a role in the pathogenesis of several human age-related diseases [25].

It is well-known that TLRs, especially TLR2 and TLR4, are involved in various autoimmune and inflammatory disorders. Such as Multiple Sclerosis (MS), Ankylosing Spondylitis (AS), Rheumatoid Arthritis (RA), Type 1 Diabetes (TID), Systemic Lupus Erythematosus (SLE), and as well as Inflammatory Bowel Disease (IBD) [19]. Given the wide-ranging impact of TLRs on innate and adaptive immunity in several disease settings, their signaling pathways arise as attractive therapeutic targets [1].

TOLL-LIKE RECEPTOR 4

Toll-like receptor 4 (TLR4) is considered one of the best-understood members of the TLRs family, who identified as the first human homolog of the Drosophila Toll gene [26], first reported by Janeway's group [27, 28]. In 1998, TLR4 was recognized as the signaling receptor for LPS or endotoxin from the outer membrane of Gram-negative bacteria [29].

Human TLR-4 is located on chromosome 9q32–q33 and contains four exons [30]. Hematopoietic and non- hematopoietic cells, including endothelial cells [31], cardiac myocytes [32], and cells of the central nervous system (CNS), express TLR4 on their surface [33]. TLR4 is located on the plasma membrane and can also be an intracellular TLR since it can be internalized and stimulate intracellular pathways [34].

The exogenous PAMPs, like LPS, taxol, viral glycoproteins, rSV fusion protein, and mice mammary tumor virus (MMTV) envelope protein, are ligands that TLR4 can recognize. Besides, necrotic cells, HSPs, high mobility group box 1 (HMGB1), fibronectin, extracellular cell matrix (ECM) components, fatty acid, minimally oxidized low-density lipoprotein (mmLDL), hyaluronic acid, β-defensin-2, amyloid peptide, and fibrinogen are endogenous ligands of TLR4 [19, 26, 35]. Recently, TLR-4 was the first identified TLR whose crystal structure was solved and led to the prediction of the mechanism of its interaction with its cognate ligands [36]. Like the rest of the family, its structure consists of three domains [26]. The extracellular Leucine Reach Repeat (LRR) domain is involved in recognition of the LPS of Gram-negative bacteria. The prototypic TLR4 ligand, the foremost ligand of TLR4, is LPS and is the main factor of the outer membrane of Gram-negative bacteria [26, 36]. The TIR domain has homology with the IL-1 receptor (IL-1R), which is responsible for the propagation of the signal within the cell [36].

TLR4 signaling can follow two different intracellular pathways. From the plasma membrane, in other words, the MyD88-dependent pathway via TIRAP induces the NF-κB and activator protein-1 (AP-1), resulting in the release of inflammatory cytokines, e.g., IL-6 and TNF-α. Alternatively, from the endosome, or the MyD88-independent pathway via TLR4 initiates the TRAM-TRIF pathway, leading to the activation of IRF3, the production of type I Interferon (IFNs), and a late wave of NF-κB activation [34, 37]. Indeed, these pathways must regulate the balance between cell survival and inflammation. TLR4 activation can induce one or more of four adaptor proteins: MyD88, TICAM1 (also known as TIR domain-containing adapter molecule 1), TIRAP, and TICAM2 [38].

Activation of TLR4 requires dimerization, which happens by molecules such as Cluster of differentiation 14 (CD14) and myeloid differentiation factor-2 (MD-2) [32]. After activation through dimerization, an internal cell cascade activates, which leads to the release of several interleukins, interferons, and other signaling substances. These signals attract macrophages, NK cells, and mast cells, which subsequently may release reactive oxygen species (ROS) and reactive nitrogen species (RNS) [39].

Activating TLR4 by LPS is a complex process in that many molecules are involved, including LPS, CD14, MD2, and LPS-binding protein (LBP) [39]. LPS is composed of lipids and carbohydrates, with a high level of structural complexity, and consists of 3 different constituents that are the O antigen, an O-polysaccharide chain of variable length; the core oligosaccharide; and lipid A, which contributes to most of the immune-stimulatory activity of the molecule [11, 26, 35, 36]. Recently, It revealed that both plasma-membrane and endosome pathways of TLR4 are required to respond to LPS fully. Additionally, TLR4 intracellular signaling boosts micropinocytosis and antigen presentation, and recently, it has also been shown to be involved in the recognition and uptake of apoptotic cells [40].

Available evidence has indicated the activation or suppression of TLR4 in the development and progression of various inflammatory diseases. Hence, TLR4 can be an excellent therapeutic target for treating inflammatory diseases [29].

Three different approaches are available for pharmacological interventions in the TLR4 signal pathway: 1- anti- LPS strategies that aim to neutralize LPS; 2- TLR4 antagonism, including the MyD88 signaling pathway; and 3- targeting inflammation and ROS/RNS [39].

MYD88

MyD88 is the most significant adaptor protein for TLR4 signaling known to hyper-activate NF-κB, MAPK, and phosphoinositide 3-kinase (PI3K) pathways driving tumor survival [41].

MyD88-mediated signaling generally occurs at the plasma membrane and involves a rapid presence of MyD88 and MAL proteins. Engagement of these adaptor molecules stimulates a range of events by phosphorylation of IRAKs, the association of TRAF6, and the downstream activation of TAK1, moderated by the adaptor proteins, TAK1-binding protein 2 and TAK1-binding protein 3 (TAB2 and TAB3). TAK1, in turn, activates the MAPKs, JNK, extracellular signal-regulated kinases (ERK1/2), p38, and the IkB kinase complex (IKK), causing the activation of imperative transcription factors, such as NF-κB and AP-1, that eventually encourage the production of pro-inflammatory cytokines [6, 42]. MyD88 and TIRAP are involved in the early activation of NF-κB and MAPK, whereas TRIF and TRAM are critical for the late activation of NF-κB and the activation of IRF-3 [43]. Noulin et al. [44] analyzed the role of TLR signaling and the impact of different cell types in response to aerogenic LPS. To illustrate this, macrophages from MyD88−/− mice are inefficient in many TLR4-mediated reactions, such as LPS-induced secretion of cytokines (e.g., IL-6, TNF-α, and IL-1β), which shows the importance of MyD88 in TLR4-mediated signaling [5].

Based on recent observations, upon TLR4 activation, TIRAP facilitates the interaction of MyD88 with TLR4 via its TIR domain, and that's where the "myddosome" formed, a giant molecular platform composed of MyD88, TIRAP, and IRAK proteins [45].

Despite the differences between MyD88-dependent and MyD88-independent pathways, both contribute to host defense and involve the immune response [46]. To illustrate, in TLR4 signaling, the MyD88-dependent way is essential for cytokine induction, such as NF-κB activation. On the contrary, the MyD88- independent pathway can lead to DC maturation [27].

Since MyD88 is involved in TLR4 signaling, it plays a significant role in infectious diseases, cancer, and autoimmune diseases, so that it can be an attractive target for intervention in these diseases [1].

TLR4 AND INFLAMMATORY DISEASES

Much evidence has shown that the activation of TLR4 results in inflammation and carcinogenesis, such as gastric cancer and human epithelial ovarian cancer [1].

The functional role of Toll-like receptors, mainly TLR4, has been proposed in diseases such as atherosclerosis [47], RA [48], allergy [49], neuropathic pain [50], ischemia/reperfusion injury [51], hemorrhagic shock [52], alcohol-induced neuroinflammation [53], and diabetes [54], asthma, cardiovascular disorder, obesity, metabolic syndrome, autoimmune disorders, schizophrenia, bipolar disorder, autism, clinical depression, chronic fatigue syndrome, and toluene inhalation [39]. The roles of TLR2 and TLR4 in psoriasis, an immune-mediated skin disease characterized by abnormal keratinocyte differentiation and proliferation, are implied by increased expression of TLR2 and TLR4 on BPMCs and keratinocytes in patients [55].

Accordingly, the expression level of TLR2 and TLR4 increased in chronic inflammatory diseases of the CNS with an autoimmune origin, such as MS and its animal model, rodent experimental autoimmune encephalomyelitis (EAE) [56].

Recent studies demonstrated that TLR2/4 could be used as an interface between innate immunity and the pro- inflammatory state of Type 1 Diabetes (T1D) because the mRNA expressional levels of TLR2, TLR4, and MyD88, as well as ligands of TLR2/ TLR4, such as Hsp60 and HMGB1, are elevated in T1D patients [57].

A significant increase in expression of the TLR4 gene was shown in Peripheral Blood Mononuclear Cells (PBMCs) of AS patients compared to healthy controls [58]. In an experimental model, Kuang et al. [59] reported that TLR4 is expressed in murine tumor cells and that the activation of TLR4 in these cells by LPS induced the expression of various factors, including IL-6. Furthermore, it has been shown that the damage to the liver through alcohol consumption can be stopped by the suppression of MyD88, a molecule involved in TLR signaling [60].

TLR4 signaling, via activation of the adaptive immune reaction, could be helpful in the chemo, and radiotherapy of cancers, as TLR4-deficient animals have shown to have an inadequate response to such therapy [61].

The activation of TLR4 signaling pathways is associated with "stress-inflammation-depression." TLR4 signaling pathway may be a possible target for the anti-inflammatory treatment of depression [62].

Recently, the link between TLR and experimental autoimmune diseases, like RA, has also become apparent. It reported that inhibition of TLR4 suppressed the severity of experimental arthritis and resulted in lower IL-1 expression in arthritic joints [63].

TLR4 ANTAGONISTS

As mentioned earlier, the proper suppression of TLR-4 signaling pathways is critical to maintaining the balance between host-defense functions and inhibition of harmful effects in autoimmune [58] and chronic inflammatory disorders [64, 65], and some negative regulators control this repression to prevent aberrant inflammatory responses [66-69].

Therapeutically, some TLR antagonists (small molecules) bind selectively to TLRs and inhibit their signal transduction and downstream signaling events in different ways. Such as neutralizing antibodies to TLR ectodomains, small molecules that block enzymes in the signaling pathway (e.g., IRAK4), and finally, agents that block protein-protein interactions in the TLR signaling cascade [58, 64].

Unfortunately, the clinical use of TLR inhibitors is limited due to the need for their available ingredients [70]. Exploring new molecules that can interfere with TLRs, their co-receptors, and their signal transduction is imperative.

One of the exogenous synthetic antagonists for TLR4 is TAK-242 (Resatorvid), a Small Molecule Inhibitor (SMIs) with an anti-sepsis effect that inhibits the communication between TLR4 and two adaptor proteins (TIRAP and TRAM), which was reported to inhibit the TLR4 signal transduction and subsequently reduce the complication in different studies [29, 58].

Other SMIs are previously developed drugs, while their suppressing effect on TLR2 and TLR4 has been recently discovered, like statins and Angiotensin II Receptor Blockers (ARBs). For instance, valsartan (a member of the ARB family) can reduce the secretion of pro-inflammatory cytokines release, while vandesartan, another member of this family, can suppress both TLR2 and TLR4 activation [71-73].

Tollip, an inhibitory protein in TLR2 and TLR4 signaling pathways, is associated with IRAK-1 to reduce IRAK- 1 auto-phosphorylation levels and inhibit its kinase activity [74, 75].

ST2825, a heptapeptide analog specifically designed to inhibit MyD88 dimerization, was reported by Loiarro et al. [76]. Also, it is specific for the homo-dimerization of the TIR domains but does not affect the homo- dimerization of the death domains [77].

Since lipid A of LPS is an excellent therapeutic target for modulating the TLR4 signaling pathway, analogs of lipid A are considered TLR antagonists. For example, Eritoran (E5564) is a synthetic lipid A analog of Rhodobacter sphaeroides, which competitively binds to the MD2 and inhibits the TLR4 signaling [32].

MiR-146a has been found to inhibit the translation of the TRAF6 and IRAK1 in the downstream signaling cascade of TLR4. It has also shown that miR-146a plays a central role as an intrinsic brake on inflammation [78, 79].

Nano-inhibitors are favorable and potent TLR inhibitors, interfering with the TLR4 signaling pathway. Lipid- modified Non-Anticoagulant Heparin Nanoparticle (NAHNP) is an intriguing self-assembling nanodevice that binds to TLR4/MD2 and prevents MyD88-dependent NF-κB pathway, has an inhibitory effect on chronic inflammation in an animal model of RA [80].

Slivka et al. introduced MD-I (a minor peptide antagonist of TLR4 signaling) linked to TLR4 and can block the interaction between TLR4 and MD2 [81].

1A6 is another monoclonal antibody interfering with MD2, a co-receptor for TLR4 activation [82]. NI-0101 is a promising anti-TLR4 antibody that acts by inhibiting TLR4 dimerization, but its function is not associated with the ligand's kind or concentration [83].

Given the fact that authentic antagonists for TLR4 are very limited, M2000 and G2013 have been identified as effective drugs in the clinical phase and besides being able to control inflammation responses.

MANNURONIC ACID

Structure and Mechanism

M2000 (β-D-Mannuronic Acid) has a low molecular weight and novel patented drug (with Patent No. of DE/102016113018.4 - PCT/EP2017/067919) (Fig. 1), with no toxicity for bone marrow, liver, and kidney. This drug has been introduced as a new NSAID with immunosuppressive and anti-inflammatory properties [70], which have been confirmed in EAE, nephritic syndrome, immune complex glomerulonephritis (ICG) and adjuvant- induced arthritis (AIA) [84-87].

M2000 is one of the Alginic acid comonomers extracted from alginate by the chemical hydrolysis method in the Immunology Department of Tehran University of Medical Sciences [70, 84].

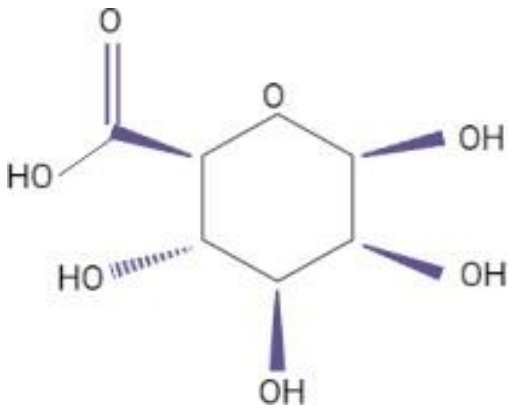

Figure 1. The chemical structure of M2000 (b-D-mannuronic acid) patented (DE-102016113018.4)

M2000, as a novel NSAID with antidiabetic, cardioprotective, and anti-tumoral efficacy, has shown excessive tolerability and safety profile with no or mild adverse events compared with many other medicines in treating different experimental and in vitro diseases [88].

As previously mentioned, due to the importance of TLRs in the pathogenesis of inflammatory disorders, inhibiting TLRs signals can be beneficial in treating such diseases [89]. Generally, because of the structure and immunostimulatory activity of alginate oligomers of M2000, this drug can act as a carbohydrate antagonist for TLR2 and 4 [90].

In Vitro And Experimental Studies

M2000, as a novel NSAID, has immunosuppressive effects on autoimmune diseases such as myelodysplastic syndrome (MDS). Treated-PBMCs by this drug showed a significant reduction of IL-6 and TNF-α as inflammatory cytokines and a significant increase in the level of Granulocyte colony-stimulating factor (G-CSF) gene expression [91].

Our previous study indicated that the activity of the cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) enzymes as primary factors in the progression of inflammatory and autoimmune diseases was strongly inhibited by M2000 [92].

In 2019 in another research, we showed that M2000 could modify oxidative stress by lowering expression levels of the Super Oxide Dismutase 2 (SOD2), Glutathione S-transferase (GST), Inducible nitric oxide synthase (iNOS), and myeloperoxidase (MPO) genes compared to the healthy expression levels, with a probable reduction of the risk of developing inflammatory diseases related to age and aging [93].

In connection with the inhibitory effect of M2000, Sharifi et al. showed that M2000 could suppress the TLR2 and TLR4 mRNA expression in HT29 cell lines. Since HT29 cells are human colon adenocarcinoma cell lines and are always used as a model of luminal surface colonic epithelial cells in vitro, It's hypothesized that this drug can reduce inflammatory responses and oxidative stress in IBD patients [94].

In 2017, Aletaha et al. showed efficient inhibition of MyD88, NF-κB, TNF-α, and IL-6 expression in HEK293 cell lines [90], and also Mortazavi Jahromi et al. at the same period showed that M2000 could modify TLR2 signaling through inhibition of the adapter molecules IRAK1 and TRAF6, the transcription factor NF-κB, miR- 146a and finally the reduction of pro-inflammatory cytokines production [95].

Tolerability and anti-inflammatory effects of M2000 have been established when administered orally in animals in various models of EAE, AIA, nephrotic syndrome, and acute glomerulonephritis [85, 96-98]. In support of this, Fattahi et al., in their preclinical study, not only found no mortality and no abnormality in clinical signs, body weight, relative organ weights, necropsy, and no significant difference in hematological, biochemical, and histopathological parameters in any of the animals in experimental models [98, 99] but also indicate that the optimum dosage of this drug, could be considered almost safe for humans [87].

According to the studies, the immunosuppressive effects of M2000 were found in animal models and cell lines [85, 100]; Also, the anti-oxidant property of M2000 and its monomer G2013 was proven in animal models [101- 103]. In the M2000-treated diabetic rats, it was found that serum glucose levels were decreased, followed by an increase in serum insulin levels. Also, a dramatic reduction in the inflammatory markers and also symptoms were seen [104].

The study on microRNAs (miRNAs) in Type 2 diabetes mellitus (T2DM) rats showed upregulating in the expression level of miR-34a in rats treated with M2000. In the same way, a dramatic decrease in expression levels of miR-126 and miR-125a-5p has been observed in those rats after treatment with β-D mannuronic acid and α-L- guluronic acid, respectively [105]. These miRNAs, especially in higher expression, are considered potential biomarkers for T2D diagnosis [106, 107].

Clinical Studies

Following the observation of the positive effects of M2000 in an animal breast cancer model [108], a clinical trial of phase II breast cancer patients found a reduction in gene expression which plays a notable role in the development of chronic inflammation, angiogenesis, tumorigenesis, and metastasis including, MMP-2, MMP-9, CCL22 and TGFβ1, and Regulatory T Cells (Tregs) frequency [109].

Accordingly, the efficacy and safety of M2000 were approved in clinical trial Phase I/II on RA. This drug has shown an inhibitory effect on Anti-Cyclic Citrullinated Peptide Antibodies (anti-CCP), Rheumatoid Factor (RF), anti-double strand DNA (anti-dsDNA), acute phase reactants, and also a reduction in IL17 and Retinoic acid- related orphan receptor γ t (RORγt) gene expression after oral administration in RA patients [110].

Oral administration of M2000 in phase III RA patients showed a reduction in the expression of miR-155 and NF- κB as long as to increase the expression of suppressor of cytokine signaling-1 (SOCS1) and SH2 domain- containing inositol-5′-phosphatase 1 (SHIP1) in treated- PBMCs in these patients [87].

The β-D-mannuronic acid significantly reduced the disease activity and physical function of patients with AS. Also, the gene expression of Myd88, IKBalpha, and NF-κB was downregulated by inhibiting the TLR/ NF-κB Signaling Pathway in these patients after treatment [58].

In 2018, Fattahi et al., in a clinical trial study, showed the effect of M2000 on inflammatory responses in phase I/ II AS patients was more impressive than naproxen and placebo. And also, the incidence of gastrointestinal and other side effects was less in patients treated with M2000 [111].

In the most recent clinical trial on patients with secondary progressive MS, Najafi et al. have shown that M2000 can downregulate IL-17, STAT1, and STAT3 genes in addition to reducing the expression of TLR2 and TLR4 on PBMCs [112].

GULURONIC ACID

Structure and Mechanism

The α-L-guluronic acid (G2013) [113] with the molecular formula ($C_6H_{10}O_7$) is a monomer of M2000, prepared from alginic acid sodium salt (Fig. 2), has advantages including its low molecular weight and natural base, immunomodulatory and anti-aging effects along with high safety property [103, 114-116].

This drug was discovered after M2000, and the purification method was carried out based on a modified procedure of the acid hydrolysis method by Nazeri et al. [114].

G2013, as an NSAID, is a novel immunosuppressive agent with no or little side effect in increasing the risk of infectious diseases and cancers [117].

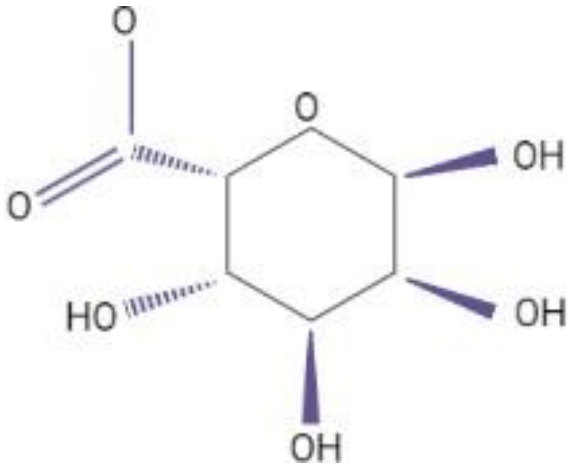

Figure 2. The chemical structure of G2013 (α-L-guluronic acid) patented (PCTEP2017067920)

In Vitro And Experimental Studies

In 2018, the researchers found that although G2013 had no profound impact on the protein expression of TLR2 and TLR4, it had an immunomodulatory effect on the TLR2 and TLR4 signaling cascade (e.g., NF-κB, IκB, and MyD88) and cytokine production by PBMCs like IL-1β [118]. It is also specified that G2013 not only can moderate the TLR4 signaling pathway by decreasing downstream signaling molecules but also has no effect on miR-146a gene expression as an anti-inflammatory factor in innate immunity (Fig. 3) [119].

Sharifi et al. in 2018 showed that G2013 significantly reduces NF-κB, IkB, and MyD88 mRNA expression and also decreases the secretion of IL-1β in human mononuclear cells [118].

Our previous study on the anti-aging and anti-inflammatory effects of the G2013 (guluronic acid) showed that this drug, as well as M2000, can reduce the genes expression of several oxidative stress (e.g., iNOS) [116], and also COX-1 and COX-2 enzymes in PBMCs [120].

A study in G2013 showed that this drug's anti-inflammatory and immunomodulatory effects lead to the induction of SHIP1, SOCS1 and reduce TLR4, MyD88, and NF-κB at the level of gene expression and decrease IL-1β as a pro-inflammatory cytokine in HEK-Blue hTLR4 cell line [4].

According to the other studies G2013, as well as M2000, can significantly reduce the Mean Fluorescence Intensity (MFI) of TLR2 and TLR4 as well as gene expression of NF-κB in Common Variable Immunodeficiency (CVID) [70, 102, 121, 122].

Subsequently, another study in 2019 revealed a significant downregulating in TLR2 and TLR4 gene expression in HT29 cell lines treated by G2013, which can exert its inhibitory effect [100].

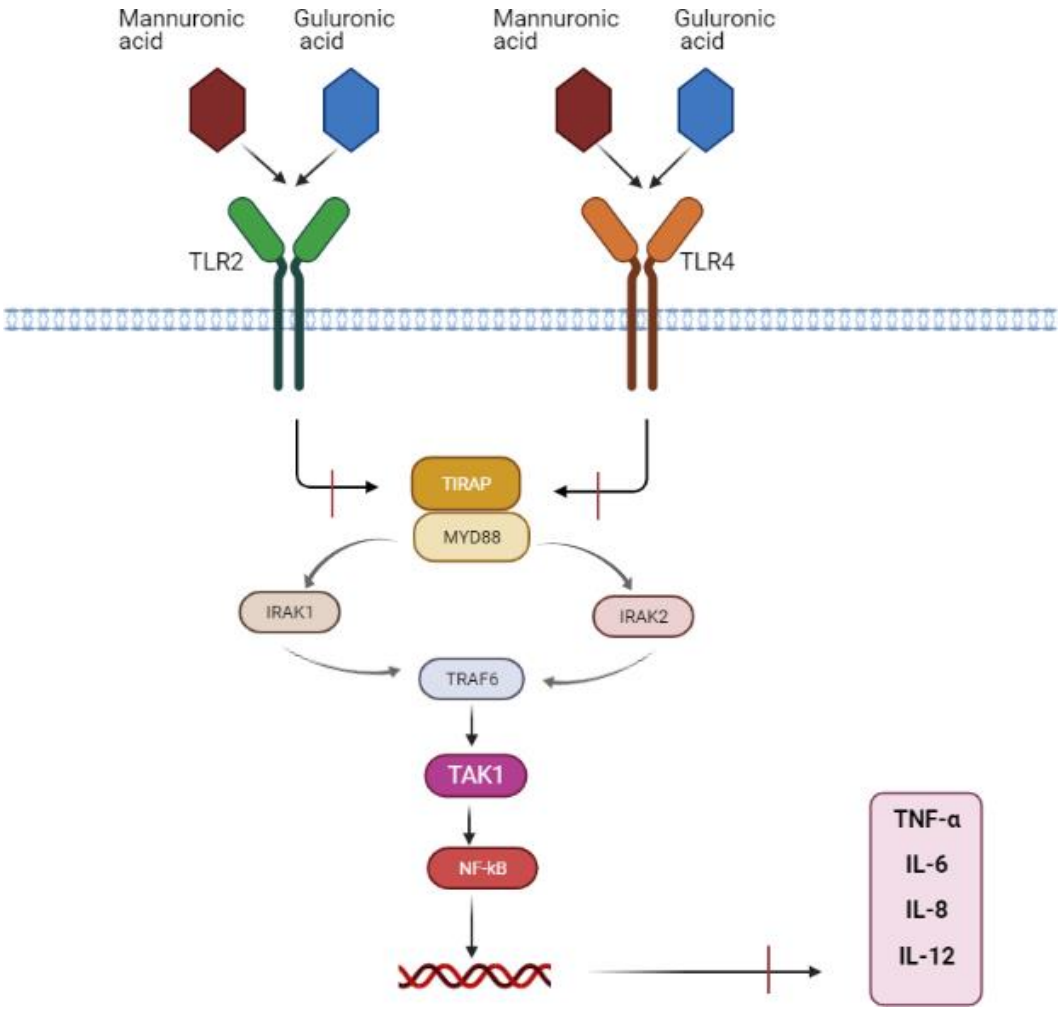

Figure 3. The β-D-Mannuronic Acid and α-L-Guluronic acid are able to affect the inflammatory pathways associated with TLR4 (and also TLR2), and consequently the reduction of inflammatory factors

Afraei et al. analyzed the model of EAE and showed that all signs of inflammation (such as serum nitric oxide (NO) levels in G2013- treated mice, were less than in the control group [123].

G2013, a potent inflammatory agent with potential anti-tumor activity, indicated that it could quickly reduce cancer-related inflammation without direct toxic effects on the cells in a murine breast cancer model [124].

PBMCs extracted from MS patients showed a decrease in the expression level of TLR2, TLR4, and TLR-α genes after being treated by G2013 [125].

Clinical Studies

The combination of G2013 with current therapy in conventional-treated RA patients has shown a positive effect, and its safety, efficacy, and tolerability are well-illustrated [126]. During a clinical study on RA patients, receiving oral administration of G2013 showed a significant increase in IL-4 and GATA-3 and a considerable decrease in RORγt gene expression after 12 weeks of treatment [127].

The investigation has shown that G2013 had a dual effect on pro-inflammatory cytokines and their transcription factors in phase I/II of RA patients. In this clinical study, a significant decrease in the level of IFNγ and Aryl Hydrocarbon Receptor (AHR) and a significant induction in gene expression of IL10 and Forkhead box P3 (FOX- P3) were seen [128].

In 2019, the study showed that oral administration of G2013 to AS patients reduced the expression levels of the RORγt, IL-17, AHR, and IL-22 and increased the gene expression levels of the GATA3, IL-4, and FOXP3. This drug can also modify the severity of articular and inflammatory signs in these patients [129].

Nazeri et al. showed that G2013 had the same effect as naproxen on AS patients and had more notable safety characteristics in identifying information than that drug [130].

Since G2013 as well as M2000, is an immunomodulatory agent which inhibits TLRs signaling, it could be considered a therapeutic target in inflammatory diseases [94].

CONCLUSION

TLRs, as an essential member of the innate immune system, recognize PAMPs and DAMPs following the activation of their signaling and triggering the inflammatory responses [1, 90].

TLR activation induces the expression of hundreds of genes, including inflammatory cytokines, chemokines, antimicrobial proteins and peptides, tissue repair, coagulation factors, and metabolic regulators. According to these facts, TLR signaling inhibition can be essential to the inflammation suppression process. TLR4, due to its close relation with inflammatory diseases, can be one of the best possible therapeutic targets in cancers and inflammatory diseases.

Since all TLR4 antagonists have acted in the preclinical phase so far, this review aims to evaluate the antagonistic, anti-inflammatory, and immunosuppressive effects of M2000 and G2013 as the only clinical antagonists on the TLR4 function. Many studies assessed whether the anti-inflammatory properties of these drugs affect the inflammation signal pathway in humans. M2000 and G2013 have been tested several times as anti-inflammatory and novel immunosuppressive agents in various experimental and clinical models [58, 70, 84, 87, 92, 94, 98, 99, 130-134].

Research has shown that the moderating effect of these novel NSAIDs can affect the inflammatory pathways associated with TLR4 (and also TLR2) and consequently reduce inflammation [4, 58, 90, 102, 118, 122].

Accordingly, M2000 and its monomer, G2013, not only prevent the progression of inflammation and reduce symptoms associated with TLR4 in experimental models of MS, AIA, nephrotic syndrome, acute glomerulonephritis, T2DM, animal Breast Cancer, and diabetic rats [85, 88, 96-99, 104, 105, 108, 118, 123] but also in clinical phase including phase I/II AS patients [58, 111, 129, 130] phase I/ II/ III in RA patients [87, 110, 126-128] in human BC [109, 124] as well as in MS patients under in vitro conditions [125] have shown positive results. These drugs cause a reduction of pro-inflammatory cytokines production related to TLR signaling and modify TLRs signaling in some cell lines by a new therapeutic approach [94, 95, 122].

Studies on these new drugs may provide important insight into the nature of the inflammatory responses and lead to the development of novel treatments for inflammatory diseases.

Declarations

- Ethics approval and consent to participate: Not applicable

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