IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Human Journals
Review Article
January 2020 Vol.:17, Issue:2

Design and Synthesis of Toll like Receptor of Inhibitors Optimizing the Potential Drugs for Autoimmune Diseases

IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
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Submission: 22 December 2019
Accepted: 28 December 2019
Published: 30 January 2020

HUMAN JOURNALS
www.ijppr.humanjournals.com

Keywords: Toll like Receptor, Inhibitors, Potential Drugs, Autoimmune Diseases

ABSTRACT

The first line of defense during infection relies on detecting the presence of pathogens and triggering pro-inflammatory responses by pattern recognition receptors (PRRs). Among PRRs, Toll-Like Receptors (TLRs) have been studied most extensively. TLRs are membrane receptors that recognize molecular patterns associated with pathogens and damaged cells to initiate immune responses (3). TLRs are good drug research targets because improper activation of TLRs has shown to be correlated with the pathogenesis of various autoimmune diseases (2). TLR8 is an endogenous receptor that recognizes single-stranded RNA from viral infections. When TLR8 is improperly activated by self-RNAs, it can lead to rheumatoid arthritis (RA) or other systemic autoimmune diseases such as systemic lupus erythematosus (SLE), systemic sclerosis (SSc), and Sjögren's syndrome(3). The recently published study of Yin Lab and Shimizu Lab has revealed that the newly-identified TLR8 antagonists inhibit TLR8 signaling through a novel mechanism. An open space in the binding pocket with the TLR8 antagonists, CU-CPT9s, suggests that their structures can be optimized(2).

INTRODUCTION

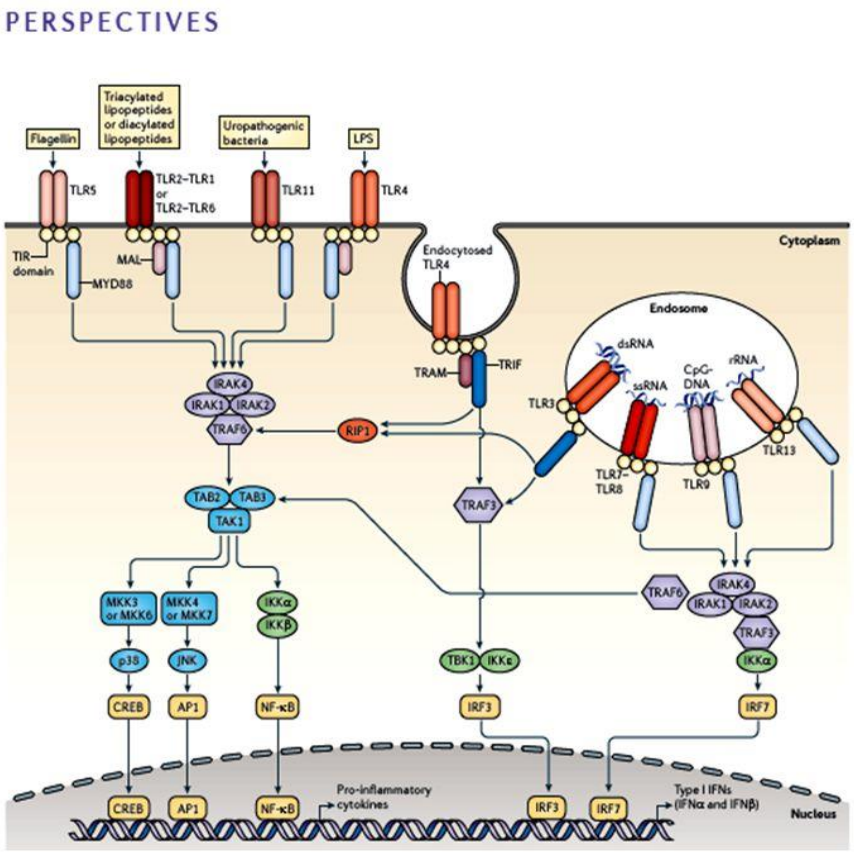
AUTOIMMUNE DISEASES

An autoimmune disease is a condition arising from an abnormal immune response to a normal body part. There are at least 80 types of autoimmune diseases. Nearly any body part can be involved. Common symptoms include low-grade fever and feeling tired. Often symptoms come and go. The cause is generally unknown. Some autoimmune diseases such as lupus run in families, and certain cases may be triggered by infections or other environmental factors. Some common diseases that are generally considered autoimmune include celiac disease,type1 diabetes mellitus, Graves's disease, inflammatory bowel disease, rheumatoid arthritis. The diagnosis can be difficult to determine.

TOLL LIKE RECEPTORS

Toll-like receptors are a class of proteins that play a role in the innate immune system. They are single, membrane-spanning noncatalytic receptors usually expressed on sentinel cells like macrophages and DCs, that recognize structurally conserved molecules devised from microbes.TLRs include TLR1, TLR13 (12 and 13 are not found in humans). During infection, the first line of defense of our immune system relies on pattern recognition receptors (PRRs) to detect the presence of pathogens and trigger pro-inflammatory responses. (1) Among PRRs, the family of Toll-like receptors (TLRs) has been studied most extensively. (1,2)TLRs are membrane receptors that recognize molecular patterns associated with pathogens and damaged cells to initiate immune responses. TLRs have shown to play an important role in mediating between the innate and adaptive immune systems. Thus, the discovery of TLRs in the late 20th century, and studies done on them since then, have allowed researchers to further the knowledge of connections between the innate and adaptive immune system.

PATHWAYS OF MAMMALIAN TLR SIGNALING



As shown in figure 1, TLR8 is located on the endosomal membrane and it recognizes single-stranded RNA from viral infections. TLR8 is mainly expressed on myeloid DCs, monocytes, differentiated macrophages, and regulatory T cells.(1,2) Although TLR7 and TLR8 both recognize ssRNA, TLR8 is only functional in humans and it is nonfunctional in mice.(3) This implies the limitation of murine model studies of TLR8. Each of the two degradation products of ssRNA, uridine, and oligonucleotide, binds to distinct sites on TLR8 to transform its conformation to the active homodimer form.(1) Then, the homodimer of TLR8 associated with the adaptor protein myeloid differentiation primary response 88 (MyD88). This triggers MyD88-dependent signaling pathway to activate NF-κB, producing inflammatory cytokines such as tumor necrosis factor (TNF) and Interleukin-1 (IL-1).14-16 TLR8 can also be activated by remnants of self-RNAs released from dead or dying cells and lead to improper activation of TLR8, which may contribute to rheumatoid arthritis and other systemic autoimmune diseases.

TLR8 and Autoimmune Diseases

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is characterized by infiltration and accumulation of activated immune cells in the synovial joints that result in cartilage and bone destruction.(4,7) The chronic production of inflammatory cytokines contributes to the RA pathogenesis, with TNF as the best-known contributor of RA.(7,2) The study done by Sacre et al. (1,7) demonstrated that suppressing cytokine production of endosomal TLRs by a non-specific inhibitor significantly reduced the spontaneous TNF and IL-6 production from human rheumatoid synovial membrane cultures. It also reported that when a range of TLR ligands was used to stimulate RA synovial cells, TLR8 induced the biggest secretion of TNF.(1,7)

Other Systemic Autoimmune Diseases

The systemic lupus erythematosus (SLE) is characterized by the loss of tolerance to self-nuclear antigens.(2,1) These self-antigens, host DNA and RNAs, are released from cell apoptosis and are normally removed rapidly by the immune system. However, SLE patients could have defects in the clearance of apoptotic cells and the autoantigens released from those cells.(2) The accumulation of autoantigen makes self-RNAs more accessible to TLR8,(1,2) which may contribute to SLE by inducing IFN-α and other inflammatory cytokines. Systemic sclerosis (SSc) is also found to be closely related to TLR8. SSc is characterized by small vessel vasculopathy, production of autoantibodies, and fibroblast dysfunction leading to increased collagen accumulation in the skin and major organs. The main cause of SSc is the deposition of excess extracellular matrix (ECM), and this can be caused when there is an imbalance of enzymes that break down ECM.(2,3) The tissue inhibitor of metalloproteinases (TIMP-1) is an inhibitor of those enzymes, and excessive production TIMP-1 is linked to TLR8 signaling.(2,4) The excess TIMP-1 from TLR8 signaling can inhibit the degradation of ECM, and this may lead to SSc. Additionally, TLR8 could be related to Jorgen's syndrome, a common type of systemic autoimmune disease that can be primary or occur in association with other autoimmune diseases.(2,5) The up-regulation of TLR8 gene expression has been observed in patients with Jorgen's syndrome, which points to a possible TLR8 function in autoimmune processes.(2,5,6) The important roles that TLR8 plays in various autoimmune diseases demonstrate the possibility of TLR8

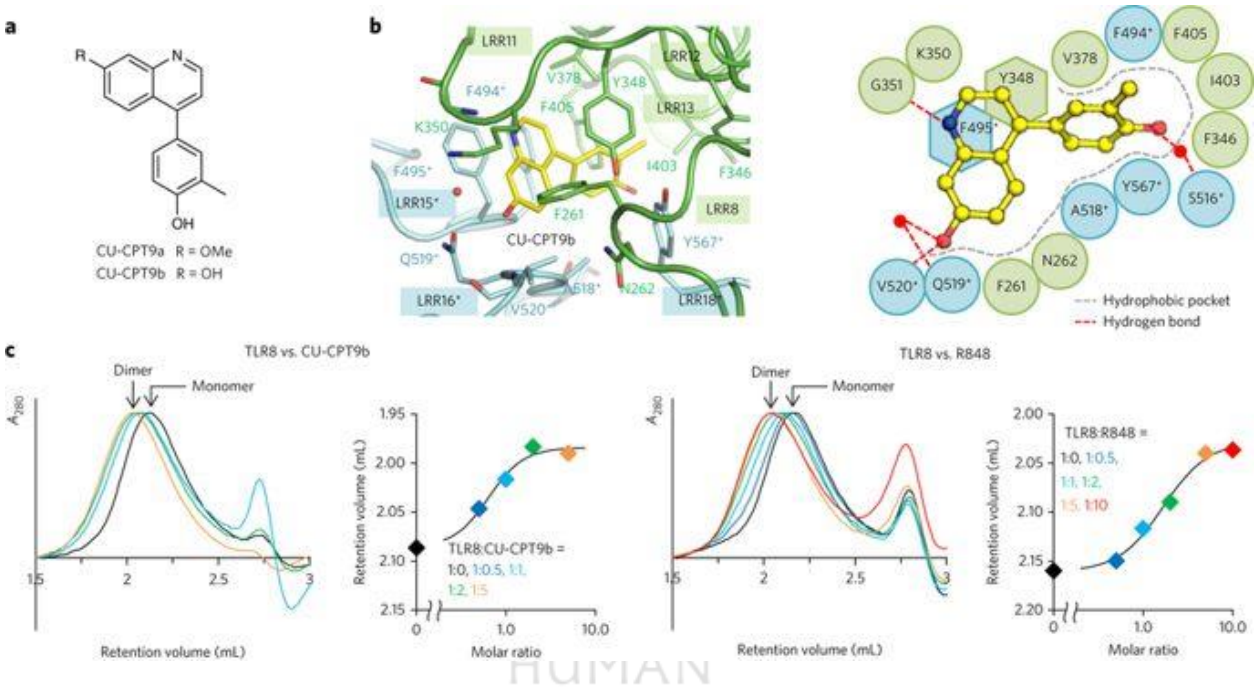
antagonists becoming effective treatments for those diseases. Thus, this highlights the significance of research in developing TLR8 antagonists. (5)

TLR8 Antagonists

Despite their therapeutic potentials, not many TLR8-specific antagonists have been identified because designing small-molecule drugs that can penetrate the outer cell membrane to interact with endosomal receptors is especially challenging. (7-9) Also, TLR8 is challenging to study because TLR8 in mice is not functional.(3) Nonetheless, in recent work of the Yin Group at the University of Colorado Boulder (with the collaboration of Shimizu Group at the University of Tokyo), a set of TLR8-selective antagonists has been identified. The TLR8 antagonists published in the literature by Zhang et al. (2,7) inhibit TLR8 signaling through a novel mechanism (Figure 3). As shown in Figure 3, the antagonist CU-CPT8m binds to a unique site between the TLR8 protomers that is close to the binding site of R848, a synthetic TLR8 agonist. The crystal structures of the antagonist-TLR8 complexes revealed that the newly-identified TLR8 antagonists stabilize the preformed TLR8 dimer in its resting state and prevent the dimer from ch of the TLR antagonist, which led to the compounds CU-CPT9a and CU-CPT9b.

(Figure 4).(2,7) These two optimized compounds have similar structure to CUCPT8m (IC50 = 67 ± 10 Overall, this project is based on the hypothesis that TLR8 inhibition by selective antagonist may help with treating damaging immune responses of autoimmune diseases. (6) nM), but much lower half-maximal inhibition concentration (IC50) values (CU-CPT9a: IC50 = 0.5 ± 0.1 nM, CU-CPT9b: IC50 = 0.7 ± 0.2 nM). Compared to CU-CPT8m, CU-CPT9s have an extra hydroxyl group, which contributes to an additional hydrogen bond with S516. The structure of CU-CPT9s can extend farther than CU-CPT8m to increase the hydrophobic interaction with the binding pocket. This also allows CU-CPT9s to form hydrogen bonds withV520, but also with Q519 (8,9). The increased intermolecular interactions between CU-CPT9s and the binding pocket may be stabilizing its binding. The antagonist binding pocket of TLR8 is not filled by the CU-CPT9 molecules. The open space in the crystal structure of the binding pocket indicates that there is an opportunity for enhancement in the potency of TLR8 antagonists. Therefore, this thesis project was aimed to further optimize the CU-CPT9 Antagonist structure. It is hypothesized that the modification of CU-CPT9 will optimize the inhibition potency of the antagonist further and will lead to a greater therapeutic value. Overall, this project is based on the hypothesis that TLR8 inhibition by selective antagonists

may help with treating damaging immune responses of autoimmune diseases.(6) optimize the inhibition PO Overall, this project is based on the hypothesis that TLR8 inhibition by selective antagonist may help with treating damaging immune responses of autoimmune diseases. (6)tenancy of the antagonist further and will lead to a greater therapeutic value The basic structure of existing TLR8 receptor antagonists to be modified.The TLR8 antagonists published in the literature by Zhang et al. (1)



Target compounds

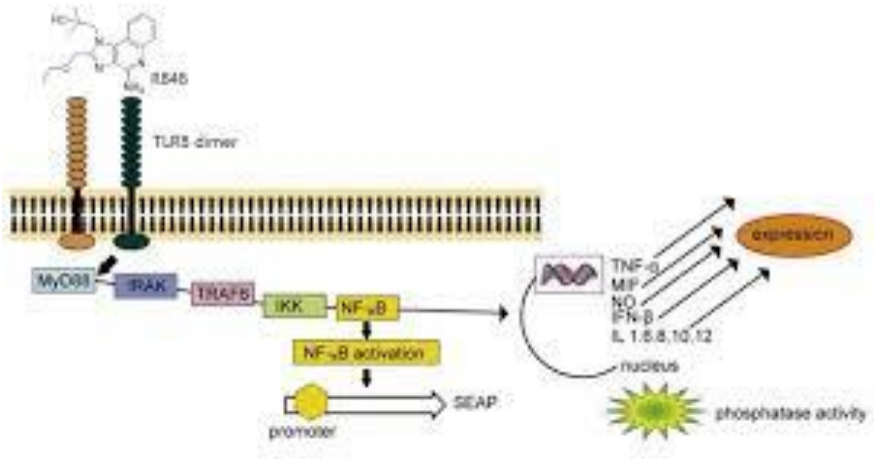
Synthesis of Target Compounds

First, compound CK1 was synthesized using the reaction steps shown in Scheme 1. The synthetic route began by creating two intermediates 1.1 and 1.2. In the first reaction step, the hydroxy group of the phenol was chlorinated using POCl3; 1000 mg/1 Equiv. of 4-hydroxy-7methoxyquinoline was stirred in 5 mL of POCl3 at 90 oC and was refluxed overnight. (3,4) The reaction was neutralized with sodium bicarbonate, and the resulting product was filtered in vacuum overnight. The Design and Synthesis of Toll-Like Receptor 8Inhibitors: Optimizing the Potential Drugs for Autoimmune Diseases obtained crude product 1.1 had 98 % product yield. In another reaction step, the boron species that was used for the Suzuki Coupling reaction in the next step was prepared; a mixture of 2-methyl-4-bromophenol (520 mg; 1 equiv.), bis(pinacolato)diboron reagent (847 mg; 1.2 equiv.), potassium acetate (1091 mg; 4 equiv.), and Pd(dppf)Cl2 catalyst (113 mg; 0.05 equiv.) was refluxed overnight at

90oC in nitrogenous atmosphere.31,32 This reaction had 76 % yield of boron product. The next reaction step combined the two products 1.1 and 1.2 through the Suzuki Coupling Reaction.31 For this reaction, a mixture of reactants (583 mg of 1.1, 705 mg of 1.2; 1:1 equiv.), K2CO3 (1248 mg; 3 equiv.), and Pd(dppf)Cl2 catalyst.

(123 mg; 0.05 equiv.) was refluxed overnight at 100ºC in the nitrogenous atmosphere. This reaction had a product yield of 32 %. The low yield could have been caused by flask not being sealed properly and some water from the atmosphere could have leaked in from that. This could have destroyed the water-sensitive catalyst. A bulk of reactants was recovered. Finally, in the last step, 4-bromo-1-butanol (10.3 µL; 1.5equiv.) was added to the 20 mg/l Equiv. of reactant in acetonitrile solution with K2CO3 (52 mg; 5 Equiv) and catalytic KI (1.3 mg; 0.1 equiv).(9,10) Unexpectedly, this reaction resulted in two products. From NMR analysis, the products were identified as CK1a (double-addition product) and CK1b (original target). Then, the crude compounds CK1a and CK1b were purified and analyzed for their potency in the next step of this project. The identity of CK1a and CK1b were confirmed by H-NMR spectroscopy (see supplementary information) and mass spectrometry (Molecular Weight: CK1a = 409.53 mol/g, CK1b = 337.42 mol/g; ESI–MS m/z:CK1a = 410.2341 [M+H]+, CK1b = 338.1764 [M+H]+). Attempts have been made to synthesize compound CK2, but they have been not successful thus far. The possible reaction steps are proposed in Scheme 2.Originally, the reaction of 4chloro-7-hydroxyquinoline with δ-valerolactone was performed in three conditions with different bases, solvents, and temperatures (pKavalues of bases: TEA = 10.75, K2CO3 = 10.25, NaH = ~35).34-36 All three conditions resulted in no reaction. Therefore, a different synthetic strategy was required, which was proposed in Scheme 2 with a dashed line. The alternative path would use a higher temperature than the condition a of TEA reaction. Also, glutaric anhydride may be used instead of δ-valerolactone because it has higher reactivity. Once the product 2.2 would be obtained, the far-end carboxylic acid on the ester chain can be selectively reduced using NaBH4 followed by (I,2) treatment, resulting in compound 2.1. (7,8) Then, the Suzuki Coupling reaction will be used to combine 2.1 and 1.2 to produce the target compound CK2.

Analysis of Compounds by Cell Culture Assays



The potency of antagonistic compounds was evaluated by cell culture assays. To test the inhibition activity of CK1a and CK1b, secreted embryonic alkaline phosphatase (SEAP) assays allow the measure of TLR8 activity by converting the TLR8 signaling product to the detectable component. Within the procedure of SEAP assay, TLR is activated by adding its synthetic ligand, which is R848 for TLR8. The activated TLR8 would then initiate a signaling cascade to activate NF-κB, which is detected by the reporter gene plasmid with an NF-κB binding site. When the reporter gene expression is increased by NF-κB, SEAP is produced. Then, the substrate is added, which will cause the color of the medium to change based on the amount of SEAP produced. The absorbance of the medium can be measured at 620nm to calculate the relative amount of TLR activation (Figure 6).(9,10) When the TLR8 antagonist is present, the level of SEAP secreted becomes lower,(7) resulting in lower absorbance. Using the concepts described above, SEAP assays were performed to test the inhibition activities of antagonists. In a 96-well plate, HEK-Blue TLR8 cells (75,000 cells/per well) in cell culture were treated in DMEM medium with 10% FBS (deactivated phosphatases). Then, the cells were treated with 1 µg/Ml R848 (Invivogen) and varying concentration of CK1a or CK1b in a separate plate (1:5 dilution, starting with 1 µM of compounds). The cells were incubated with compounds and R848 at 37°C for 24 hours. After incubation, 20 µL of culture media were transferred to a new 96-well plate and 180 µL of Quanti-Blue (Invivogen) was added to each well. The plate was incubated at 37°C again until the color change was observed (0.5-1 hours). Using a plate reader, the absorbance of the plate was measured at 620 nm.27 On each plate, there were three replicate wells with the same concentration of the compounds, and the plate was replicated three times. Data were

averaged and normalized with the absorbance of R848-treated cells as 100% activation and that of untreated cells as 0% activation (Figure 7). The IC50 values of the compounds were 3.67 ± 0.31 nM for CK1a and 0.152 ± 0.033 nM for CK1b.(7,3).

WST-1 Assay

The toxicity of compounds was also evaluated by measuring cell viability in WST-1 (water-soluble tetrazolium salt) assays. WST-1 essay is a type of tetrazolium reduction assays that measures metabolic activity First, compound CK1 was synthesized using the reaction steps shown in Scheme 1. The synthetic route of cells as a marker of viable cells. (4,1) The viable cells can readily reduce tetrazolium salt into the colored Formosan product, and the number of viable cells can be measured by recording the change in absorbance at 450 nm. (2,7, 4,1) To evaluate the toxicity of compounds, plates were prepared as described above for SEAP assay. A 40μL of DMSO was added to the half of cell-only wells to induce cell death. After incubating the plate, the supernatant was removed and 10 μL of WST-1 reagent (Roche) was added to the well. The plate was incubated at 37 °C again until the color change was observed (0.5-1 hours). Using a plate reader, the absorbance of the plate was measured at 450 nm. (2,7) Data were averaged and normalized with the untreated cells as 100% survival and with DMSO-treated cells as 0% survival (Figure 8). There was no significant decrease in cell viability with the increase in concentrations of CK1a and CK1b; thus, no toxicity was observed for both compound. [3,7]

CONCLUSION

The newly synthesized compounds ck1a and ck1b are found to be the optimized structures of CU-CPT9 TLR 8 antagonists. As mentioned, the synthesis of target compound CK2 has not been successful. If the proposed reactions in Scheme 2 succeed, the obtained compound CK2 needs to be analyzed. If the reactions fail, a new synthetic route for CK2 needs to be thought out by adjusting the possible causes of reaction failures. Once CK2 is obtained, IC50 value and toxicity of CK2 should be evaluated. If the IC50 of CK2 is not significantly different from the of CK1b, the compound's potency may be nearly optimal IC50.

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