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Miniperspective

Small Molecule Modulators of Toll-like Receptors

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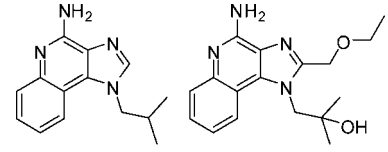
Introduction

The human immune response is mediated through two parallel immune components. The innate immunes system responds to pathogens and abnormal cells through multiple cell types including dendritic cells, macrophages, neutrophils, and natural killer cells and represents a first line of defense in mammals. The adaptive immune response system responds to pathogens and abnormal cells through the T cell and B cell systems, neutralizing the these components with T-cell receptors and antibodies respectively. Toll-like receptor (TLR[a]) functions are activated as part of the innate immune response system recognizing macromolecular molecular components of microorganisms. These structures are the pathogen-associated molecular patterns (PAMPs). Subsequent to activation the innate immune system triggers the production of cytokines and chemokines and induces the adaptive immune system. The action of vaccines is due, in part, to the activation of the TLR system. Originally discovered in fruit flies the TLRs were found as an entire family of receptors in humans. The basic biology and clinical potential of TLR based therapeutics have been extensively and recently reviewed.[1,2] These reviews provide a broad perspective on TLRs including mechanisms of action, therapies based on novel biologics, and an analysis of ongoing clinical trials.

Agonists of the TLRs would be immune system enhancers and have been proposed to be useful in the treatment of cancer and infectious diseases. Antagonists, on the other hand, are thought to have a therapeutic role in suppressing overactive immune responses, as occurs in chronic inflammatory and autoimmune diseases. Eleven TLRs have been identified, 10 of which have been found in humans. Figure 1 illustrates the cellular localization and natural ligand for the 10 human TLRs. TLR2, TLR4, TLR7, TLR8, and TLR9 have been the targets for small molecule drug discovery efforts. This Miniperspective will focus on the medicinal chemistry of small molecule TLR modulators. This is a very new field, and as such, much of the current state of the art is within the published patent filings of both large and small pharmaceutical companies. Reporting of biological activity is complicated by the fact that direct binding assays for the TLR family have not been used and SAR, when it exists, is from a variety of reporter gene or functional cell based assays. The goal of this Miniperspective is to illustrate the small molecule chemotypes that have been identified as TLR modulating agents, analyze when possible the structure−activity relationships, and place this new and emerging field of medicinal chemistry in context.

History

First discovered in the 1980s the imidazoquinoline amines such as imiquimod and resiquimod are now known to be the first small molecule immune response modulators that function through the TLR receptors.



Imiquimod Resiquimod

Imiquimod (Aldara) was introduced as an antiviral by Riker Laboratories (later 3M Pharmaceuticals) and was approved as

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[a] Abbreviations: CpG, cytosine phosphate guanosine; dsRNA, double stranded ribonucleic acid; HCV, hepatitis C virus; IFN, interferon; LPS, lipopolysaccharide; μM, micromolar; MyD88, myeloid differentiation factor 88; nM, nanomolar; NF, nuclear transcription factor; PAMP, pathogen associated molecular patterns; PBL, peripheral blood lymphocytes; PBMC, peripheral blood monocytes; SAR, structure−activity relationship; ssRNA, single stranded ribonucleic acid; TIR, toll interleukin-1 receptor homologous domain; TLR, toll-like receptor; TNF, tumor necrosis factor.

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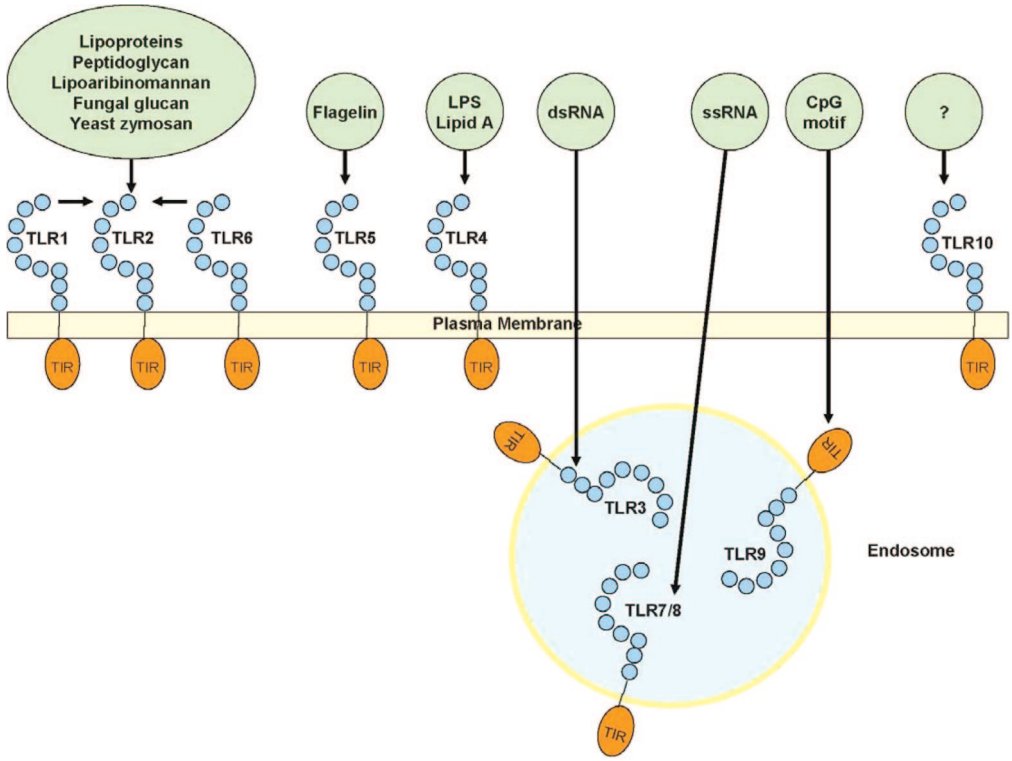


Figure 1. Cellular localization and natural ligands of the 10 human toll-like receptors. TIR is the conserved toll interleukin-1 receptor domain. TLR2 signals either alone or in association with TLR1 or TLR6.

a topical treatment for papillomavirus infection in 1997. Resiquimod was being developed as a second generation drug, but its development was suspended in 2003. The mechanism of this antiviral effect ascribed to these drugs was unknown at the time of invention and introduction.

In 2001 S. Akira and co-workers submitted a manuscript to *Nature Immunology* in November, which was subsequently published in February of 2002 and identified the mechanism of action of imiquimod and resiquimod as activators of the TLR7 pathway.[3] Simultaneously with this submission, 3M Innovative Properties filed patents that described methods of identifying compounds that activate TLR mediated cellular pathways.[4] In doing so 3M provided examples of TLR6 and TLR7 immune response modulators that spanned the entire intellectual property space of the 3M antiviral compounds previously claimed in a large number of broadly filed patents.

Akira showed that TLR7 responses are mediated in macrophages through a specific pathway identified as MyD88, which leads to the production of inflammatory cytokines. The imidazoquinolines are able to elicit cytokine production in wild type macrophages but are inactive in MyD88 deficient macrophages. The evidence was further augmented with experiments in vivo. Resiquimod induced an immune response, as assessed by levels of IFN-a, TNF-a, and IL12 in wild type mice but not in MyD88 knockout mice. The field of small molecule modulators of TLRs, which did not exist prior to 2002, came into sharp focus as a result of this publication. It is this specific area that will be reviewed in this Miniperspective.

TLR7 Agonists

Jones and co-workers have disclosed TLR7 active agents that are useful in treating viral infections and inflammatory diseases and cancer.[5,6] These efforts in toll-like receptors have been reported in two patent applications claiming TLR7 activity for purine based immune response modulators. Pfizer has reported TLR7 activity in a coupled PBL/HCV assay in which an interferon secreting cell line is co-incubated with and HCV replicon. ED_{50} values are reflected in the ability of a TLR7 modulator to increase sufficient interferon secretion to cause a 50% depletion in the HCV replicon levels. Two chemotypes have been reported (Table 1). A pyrimidinyl-imidazolone series (**1**) appears to be more potent with five examples ranging in potency from 5 to 72 nM. A second series of pyridinyl-imidazolones (**2**) was also disclosed with 38 compounds with biological activity ranging from 100 to 3260 nM.

Chong has also discovered molecules that target TLR7 to be used as antiviral agents.[7] They have reported (Table 1) multiple examples of oxo (**3**) and thio (**4**) analogues of stable nucleotide mimetic phosphinic and phosphonic acids. No specific SAR results have been reported; however, compounds are presented to be agonists in a luciferase reporter gene and to stimulate interferon release in PBMC (peripheral blood mononuclear cell) cell based assays with EC_{50} activity in the range of 10−1000 nM.

Cook et al. also disclosed 8-oxoadenine derivative as agonists in a TLR7 reporter gene assay.[8] Four compounds had reported activities below 20 nM with two of these examples listed in Table 1. These compounds are for the treatment of infectious diseases, inflammatory and allergic diseases, and cancer. While the oxoadenines discussed in Table 1 have been targeted to TLR 7, the compound class may also have some activity on adenosine AI receptors as well.[9]

Researchers at the University of California at San Diego interested in treatments for HCV patients have developed the SAR of a series of 8-amino-9-benzyladenines.[10] These scientists experimented with replacement of the 8-oxo motif seen previously with an 8-aza motif (Table 2). Simple substitution of the nitrogen for oxygen led to loss of activity (**7**). However, alkylaza (**8**, **9**) substitution restored activity in the limited examples

Table 1. 8-Oxoadenine TLR7 Agonists

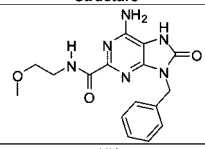
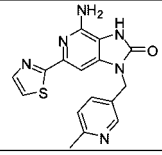
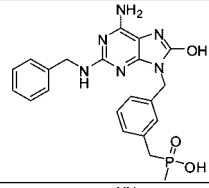
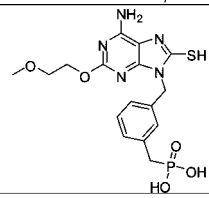
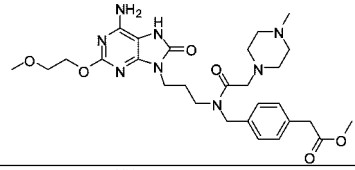
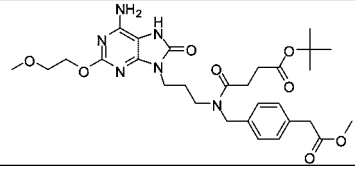
Compound	Structure	EC_{50}
1		5 nM
2		100 nM
3		10 – 1000 nM
4		10 – 1000 nM
5		< 20 nM
6		< 20 nM

Table 2. 8-Aminoadenine Agonists of TLR7

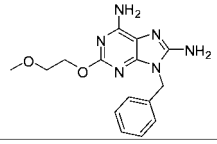
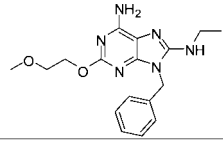
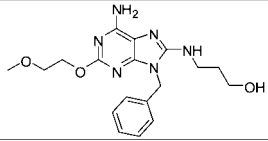
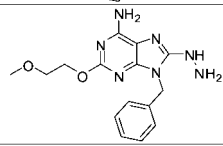
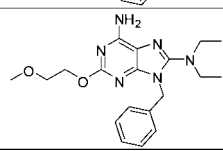
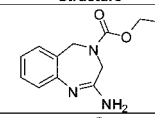
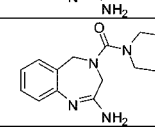
Compound	Structure	EC_{50}
7		> 10 μM
8		4.4 μM
9		3.2 μM
10		0.79 μM
11		Inactive

Table 3. Benzodiazepine TLR8 Agonists

Compound	Structure	EC_{50}
12		< 100 μM
13		< 100 μM

presented. Disubstitution (**11**), however, resulted in a complete loss of activity.

TLR8 Agonists

Doherty and Jones have discovered a narrowly focused group of 8-aminobenzodiazapines as TLR8 agonists in both a reporter gene assay and a TNF-α secretion assay in PBMC's.[11] Biological activity SAR is not presented in the patent application with the target compounds only listed as more potent than 100 μM in both assays (Table 3).

TLR9 Agonists

Yu et al. from have Idera Pharmaceuticals have published an extensive exploration of the SAR of synthetic dimeric phophorothioate 11-mer oligonucleotides, linked with a glycerol bridge, which incorporate unnatural synthetic CpG motifs.[12] Eleven deoxycytosine and twelve deoxyguanosine analogues were permuted at positions 6 and 7 of the 11-mer sequence (Figure 2). Twenty-four analogues are analyzed for their activity to (1) induce NF-kB in HEK293 cells, (2) activate cytokine production in human PBMCs, (3) activate the proliferation of human B cells, and (4) stimulate cytokine production in vivo in mice following sc administration. These synthetic oligonucleotide analogues demonstrated a complex SAR that clearly suggested that TLR9 can recognize C or G modifications in the induction of immune responses.

Jurk and co-workers also have studied the immune stimulatory effects of base modified synthetic phophorothioate linked oligonucleotides.[13] Analysis of activity was measured by the ability to stimulate lucifierase activity in a reporter gene assay for TLR9. Chemical modifications of both the cytosine motif and the guanosine were introduced and the effect of the changes evaluated within the complete oligonucleotide (Figure 3). Compound **B** showed the highest stimulatory activity when compared to oligonucleotide with unmodified bases, compound

d(5'-TCTGTC_XG_YTTCT)-$OCH_2CH(OH)CH_2O$-(TCTTG_YC_XTGTCT-5')

Figure 2. Structural template for Idera Pharmaceutical oligonucleotide TLR9 agonists.

5'(TGTC-Pu-TTTTTTTTTTTTTTT)3'

Compound A Pu = Guanine

Compound B Pu = 6-Thioguanine

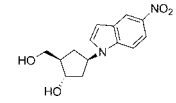
Compound C Pu = 

Figure 3. Coley Pharmaceutical oligonucleotide TLR9 agonist structures.

Table 4. Disubstituted Quinazoline TLR9 Antagonists

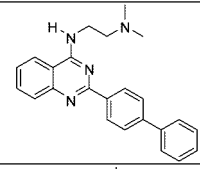
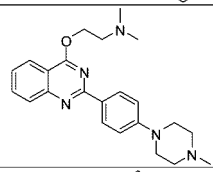
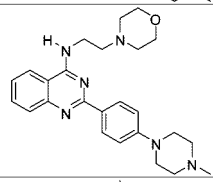
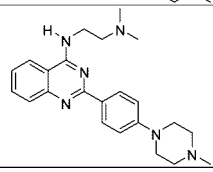
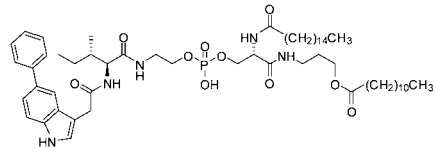
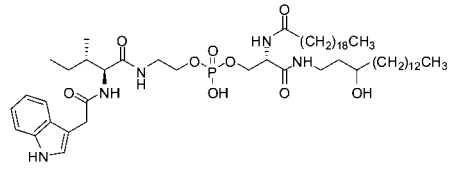
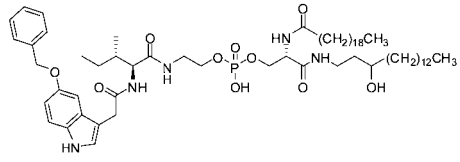
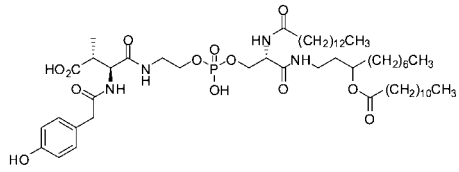
Compound	Structure	IC_{50}
14		5.2 μM
15		2.4 μM
16		0.02 μM
17		0.003 μM

Table 5. Lipopeptide Mimetic Inhibitors of TLR2

Compound	Structure	IC_{50}
18		0.23 μM
19		0.46 μM
20		0.24 μM
21		0.90 μM

A, whereas compound **C** was more potent but showed a lower overall level of stimulation.

TLR9 Antagonists

Lipford has filed extensive patents on the TLR9 antagonist activity of substituted quinazolines.[14,15] TLR9 antagonists are claimed to be useful in treating diseases that are characterized by an unwanted immune response. Diseases of this type include autoimmune diseases, transplant rejection, and sepsis. Biological activity and SAR are disclosed for a very large number of antagonists. The reported compounds were evaluated in TLR9 transfected cells and were shown to block the response to agonists (CpG oligodeoxynucleotide) induced release of TLR9. Table 4 shows examples of key elements of the SAR of these quinazoline antagonists. Compound **17** combined the best elements from the SAR to give potency in the single-digit nanomolar range.

Table 6. TLR2 Inhibitor Lipolanthionine Analogue

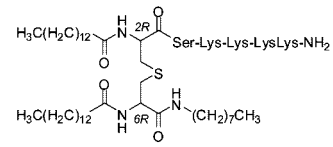
Compound	Structure	IC_{50}
22		5 μM

Table 7. TLR4 Inhibitor M62812

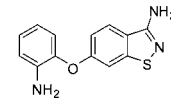
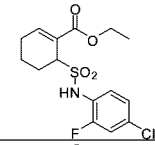
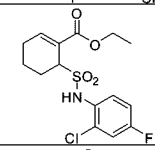
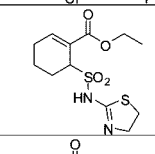
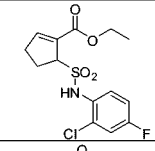
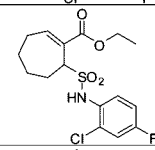
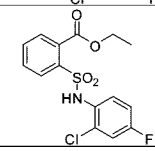
Compound	Structure	IC_{50}
23		7 μM

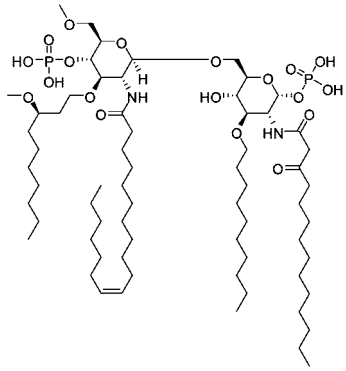
Table 8. Antisepsis Agents Active on TLR4 Mediated Cytokine Production

Compound	Structure	IC_{50}
24		160 nM
25		3.2 nM
26		>10000
27		110
28		240
29		>10000

TLR2 Antagonists

TLR2 stimulation is associated with inflammatory conditions such as pathogen induced chronic joint disease and sepsis.

Table 9. E5564: TLR4 Inhibitor Clinical Candidate

Compound	Structure	IC_{50}
30		1.5 nM

Chow, Spyvee, and co-workers, in a medicinal chemistry study and a U.S. patent, showed that novel phopholipopeptides are antagonists of TLR2 when assessed in TLR2 transfected cells as well as in human PBMC's.[16,17] Two related chemical series are illustrated in Table 5. The tryptophan series (**18**, **19**, **20**) is the more potent series with all reported compounds having activity less than 1 μM. The tyrosine series is less potent with the best compounds in the 1 μM range and upward into 20 μM. Most of the compounds reported are not selective for TLR2 over TLR4. Within the tryptophan series, however, compounds with a free right side hydroxyl (**19**, **20**) instead of an ester (**18**) are more than 25-fold selective for TLR2 over TLR4.

Researchers at the University of Tubingen have investigated the SAR of lipolanthionine (Table 6) analogues as inhibitors of TLR2 mediated secretion of IL-8 in THP-1 cells. TLR2 antagonists have the potential for the treatment of acute and chronic inflammatory conditions.[18] Twenty-three analogues were synthesized and evaluated in cultured THP-1 cells. An example of a typical compound is shown in Table 6. The chain length of the fatty acid moiety was very important for activity, with the myristic acid derivative shown having the greatest inhibitory activity. The length of the aliphatic amine, however, had little effect. Chirality at the 6-α position was critical as *R*, while the configuration at the 2-α position did not influence activity. Evaluation of the compounds as inhibitors of TLR4 mediated IL-8 secretion indicated that the compounds were not selective for the TLR2 manifold.

TLR4 Antagonists

While investigating potential therapeutics for treating sepsis, Nakamura et al. have reported that M62812 blocks TLR4 mediated signal transduction but do not develop any SAR.[19] Compound **23** has an IC_{50} in a TLR4 reporter gene assay of 7 μM and IC_{50} in PBMC cells of 3 μM. In vivo activity in mice models of inflammation and septic shock was dose dependent and had significant activity at 20 mg/kg upon intravenous administration (Table 7).

Yamada, in 2005, has reported on a novel chemical series that are inhibitors of NO and cytokine production. Starting with a screening lead (**24**, Table 8) which inhibited cytokine production in mouse macrophage cell culture, the SAR was systematically developed.[20] Extensive variation of the aniline aryl substitution was optimal with 2-chloro, 4-fluoro (**25**), heteroaryl motifs (**26**) were inactive. A variety of esters examined were active between 3 and 300 nM, with ethyl being the most active. Cyclohexenyl was the most active saturated ring system; smaller (**27**) and larger (**28**) aliphatic rings were less active. Phenyl (**29**) replacement of the cyclohexenyl was completely inactive.

The most active compound **25** (TAK-242) was shown to be an inhibitor of TLR4 stimulated cytokine release but not TLR2 stimulated cytokine release.[21] TAK-242 is active in mouse models of endotoxin septic shock when administered intravenously at doses between 0.1 and 3 mg/kg. TAK-242 advanced to phase III clinical trials for sepsis advancing directly out of successful phase I trials, although a recent check of the Takeda product pipeline no longer shows clinical activity for this drug.[22]

Eisai has introduced E5564 into the clinic for the treatment of sepsis, and it has advanced to phase 3. This drug was originally discovered in 1996 as one of an 11-member series of synthetic lipid A analogues patented for the treatment of sepsis.[23] The analogues were evaluated in vitro by the inhibition of LPS induced production of cytokines in HL-60 cells. The IC_{50} values ranged from 1.5 to 159 nM, with the most potent compound E5564 (**30**) shown in Table 9. While the mechanism was unknown at the time, subsequent published research determined that the inhibition of the endotoxin response by E5564 was due to TLR antagonist activity.[24]

Future Prospects

The developments, presented in this Miniperspective, in the field of small molecule TLR modulators have demonstrated several principles that suggest an important future for this area. (1) Small molecules can effectively modulate the activity of receptors that have evolved to bind the macromolecular components of pathogens. (2) TLR modulators can demonstrate regular structure−activity relationships and can achieve high potency. (3) Drugs based on modulation of TLRs have achieved significant therapeutic potential as demonstrated by imiquimod. The large number of human receptors and, to date, the relatively small number of demonstrated small molecule modulators make it difficult to predict even the near-term future of this emerging field in medicinal chemistry. There will be, however, very significant opportunity to develop breakthrough therapies via the TLR related pathways.

Biography

Michael Czarniecki is currently Senior Director of Chemical Research and Chemical Technologies at Schering-Plough Research Institute, which he joined in 1981. His major responsibilities include medicinal chemistry, HTS screening file management, compound library synthesis, chemistry outsourcing, and cheminformatics activities. Dr. Czarniecki completed a Ph.D. at the University of Pennsylvania studying the biophysical chemistry of cell surface carbohydrates and was a National Science Foundation postdoctoral fellow at Columbia University where he did research in bioorganic chemistry with Professor Ronald Breslow.

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