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Therapeutic Developments Targeting Toll-like Receptor 4 Mediated Neuroinflammation

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Abstract

Toll-like receptors (TLRs) have been shown to play an important role in the immune system, which warrants their remarkable pharmacological potentials. Activation of TLRs requires participation from specific PAMPs (pathogen associated molecular patterns) and accessory proteins such as MD2 (myeloid differentiation protein 2), LBP (lipopolysaccharide binding protein), and CD-14. Assembly of the TLR4-MD2-LPS complex is essential in TLR4 activation. Recent studies have revealed that TLR4 activation is a significant trigger of signal transmission pathways in the nervous system, which could result in chronic pain as well as opioid tolerance and dependence. Researchers of the molecular structure of TLRs and their accessory proteins have opened a door to syntheses of TLRs agonists and antagonists, such as Eritoran. Small molecule modulators of TLR4, such as MD2-I and tricyclic anti-depressants, offer more promising prospects than peptides for their convenient oral usage and lower cost. We mainly discuss the mechanism and clinical prospect of TLR4 agonists and antagonists in this review.

Graphical Abstract

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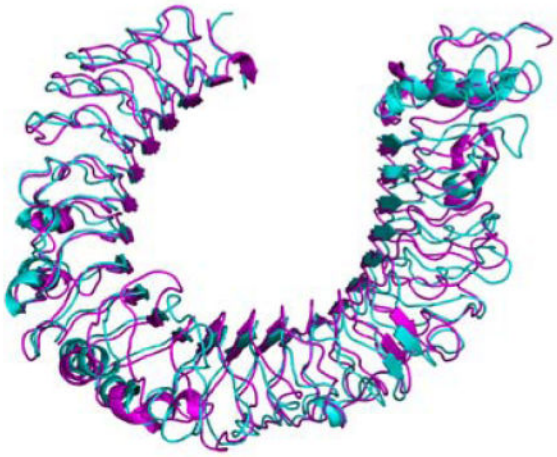
Conflicts of interest

The authors declare no conflict of interest.

Author Contributions

All authors made substantial contributions to the conception and design of this study. All authors read and approved the final manuscript.



Toll-like receptors (TLRs) are a family of membrane proteins crucial to the cellular innate immune response. Pain is one of the most intractable and widespread conditions that people face today. At the crossroads of the immune system and pain is TLR4. This review aims to provide a brief summary about TLRs and how they their signaling affects pain. Furthermore, this review also takes a look at the molecules that are able to modulate TLR4 signaling as potential drug candidates.

Keywords

Toll-like receptor; inflammation mechanism; neuropathic pain; analgesia; accessory protein; agonist; antagonist; small molecule modulator; drug discovery

1. Introduction

Since ancient times, the secrets of the body's self-defense system have been of interest to the earliest doctors. Centuries later, much of the human immune system has been explained, while some details still remain unsolved. Toll-like receptors (TLRs) are one of the greatest discoveries of immune science, and have been widely known to play an important role in the inflammation response [1] Inflammation is one of the most significant immune reactions, triggered by the binding of specific ligands called PAMPs (pathogen associated molecular patterns) to their respective TLRs [2]. As one of the earliest discovered pattern recognition receptors (PRRs) [3], the TLRs were named after the toll receptor of *Drosophila*, because of their structural similarity and function in host defense [2]. Binding of specific ligands with TLRs (Table 1) will initiate a particular signal transduction pathway, whose activation would lead to the expression of specific effector genes (Figure 1) [4]. Macrophages would also be activated upon recognition of the pathogen signals [5].

1.1 Ligands of TLRs

TLRs make up one of the biggest family of PRRs. There are at least 13 Toll-like receptors that have been discovered in mammals [6]. Among them, TLRs 1-10 have been found

functioning inside the human body [2]. The different members of the TLR family can recognize diverse ligands shown in Table 1 [2, 7].

1.2. TLRs in the development of immune diseases

While TLRs play a protective role as an integral part of the innate immune system, misidentification or excessive activity of TLRs may lead to chronic immune diseases such as osteoarthritis, asthma, neuralgia and sepsis [8]. The inappropriately activated signaling pathway will result in either attack of immune cells, such as macrophage or dendritic cells, or apoptosis of healthy cells [9].

2. TLR4-mediated neuroinflammation

2.1. TLR4 signaling pathway

In the study of TLR4 and its signaling pathway, a route diagram of the TLR4 signaling pathway in microglia was clearly developed [9a]. There are several important processes prior to TLR4 activation, which is triggered by a PAMP (usually LPS) or danger associated molecular pattern (DAMP) molecule. Firstly, with the assistance of LPS-binding-protein (LBP), LPS is extracted from the membrane structure of the pathogen or damaged cell [10]. Then, LBP transports an LPS molecule to cluster differentiation antigen 14 (CD14) to form a dimer complex (soluble CD14-LPS)$_2$ or monomer complex (membrane CD14-LPS). Either complex can deliver LPS to the MD2-TLR4 heterodimer complex (MD2, myeloid differentiation protein 2, a TLR4 specific accessory protein) [11]. The interaction between those complexes results in the formation of a new ternary complex (TLR4-MD2-LPS)$_2$ (Figure 2) [12]. The formation of (TLR4-MD2-LPS)$_2$ triggers two intracellular signaling pathways, which results in the production of pro-inflammatory cytokines [13]. Those two signaling pathways begin with the binding of intracellular adapter proteins, MyD88 (myeloid differentiation primary-response gene 88) or TRIF (Toll/IL-1R (TIR)-domain-containing adaptor protein) (Figure 1) [14].

The specific details about how the previously mentioned cytokines and signaling proteins bind or react with each other are not completely understood [14a]. Studies have shown that one of the final products of those signaling pathways is nitric oxide (NO), which is generally recognized as an important neural signaling molecule. For this reason, the detection of NO concentration has become a popular method to evaluate the activity of TLR4 [4].

2.2. TLR4 activation in pain

Recent studies of chronic immune diseases have indicated that development of chronic pain is closely associated with the activation of TLR4 [9a]. Chronic pain is one of the most intractable diseases with high prevalence. Recently, a systematic review containing 13 studies demonstrated that the prevalence of chronic pain in developed countries may be as much as 55% [15]. Chronic pain is pain without biological value that has persisted beyond the normal tissue healing time (usually considered to be 3 months), according to the International Association of the Study of Pain (IASP) definition [15]. Usually chronic pain is due to the physical damage of specific neural cells [15]. Emerging evidence has demonstrated that TLRs would not only respond to the PAMPs of pathogens, but also could be activated

by DAMPs [16]. DAMPs include molecules released by damaged cells during apoptosis, and other endogenous ligands which are involved in cell apoptosis, such as heat shock proteins, extracellular matrix degradation products, protein HMGB-1 *etc* (Table 1) [16–17]. These findings connect the neural pain to the activation of TLRs.

Expressions of TLRs are found in diverse components of the nervous system such as microglia, astrocytes, oligodendrocytes, Schwann cells and neurons (Table 2) [16, 18]. Unlike TLRs of the immune system, these specific TLRs, especially TLR4, may release different kinds of signaling molecules after activation (Figure 1) [19]. Acting as macrophages in the nervous system, microglia may help to eradicate the damaged neural cells and invasive pathogen in the central nervous system (CNS) [16, 20]. On the other hand, release of signaling molecules at the end of the TLR signaling pathways would promote the inflammatory reaction inside the nervous system, which could result in neuralgia [21]. Although various types of TLRs mRNA are found in microglia, only a part of mRNA is eventually translated into protein, mainly those of TLR4 [16, 22].

2.3. The opioid effect on TLR4

Opioids, such as morphine, are widely used to treat pain. But long-term usage of opioids may lead to opioid tolerance or dependency [23]. The analgesic effect of opioids is thought to be transmitted through the opioid receptors (such as μ, κ, and δ receptor) in a stereoselective manner. However there is another non-stereoselective opioid activation that exists, which is not well understood (Figure 3) [24]. Recent studies suggest that non-stereoselective activation of opioids is related to the activation of glial cells and TLR4 [23].

TLR4 activation could decrease opioid efficacy, and is linked to the development and maintenance of neuropathic pain [21b]. Both bio-based and *in silico*-based experiments have shown that opioids could activate TLR4 on glial cells [25]. Their activation could result in the synthesis of nociceptive cytokines, which might exacerbate neuropathic pain and counteract the analgesic effects of opioids [26]. Different from the classical opioid receptors, TLR4 is activated by the morphine metabolite morphine-3-glucuronide (M3G) instead of morphine-6-glucuronide (M6G) [24]. The most recent studies from the Watkins group show that both estradiol-3-glucuronide (E_2-3-G) and estradiol-17-glucuronide (E_2-17-G) can enhance neuropathic pain [27]. *In silico* docking was able to predict that E_2-3-G and E_2-17-G could bind to MD2 in the TLR4-MD2 complex. A cell based, secreted embryonic alkaline phosphatase (SEAP) reporter assay was employed to showcase that this binding resulted in agonistic activity. Finally, an *in vivo* experiment using Sprague-Dawley rats in a von Frey test showed that both E_2-3-G and E_2-17-G caused enhanced pain, and that the potent antagonist, LPS-RS (the LPS found in the bacteria *Rhodobacter sphaeroides*), was able to reverse the effects.

Further studies with the MD2 X-ray crystal structure suggested that the activation effect of opioids on TLR4 might be related to the LPS-mimic structure of opioids and the LPS binding junction part of MD2 [28]. Opioids can mimic LPS and bind to MD2 as an LPS substitute to activate TLR4 directly. Therefore opioids may activate TLR4 without participation of LPS [29]. However Yin and co-workers have taken morphine to study the neuroinflammation activated by opioids. In this previous work, the binding experiment of

morphine and MD2, morphine demonstrated a strong binding ability to MD2, and its binding interaction was observed to compete with LPS-MD2 binding [18g]. Further experimental results suggest that not only can morphine bind to MD2 as LPS does, but the morphine-MD2 complex can also induce TLR4 oligomerization, just as the LPS-MD2 complex does [28a, 29]. TLR4 oligomerization induced by the morphine-MD2 complex would lead to the formation of the TLR4-MD2-morphine complex, which would trigger the downstream pro-inflammatory cascade reactions [28a, 29]. These findings lead to the assumptions that TLR4 activation may aggravate pain, TLR4 may play an important role in opioid-induced hyperalgesia, and TLR4 may function against the acute or chronic analgesic effect of opioids [24]. The total of these works suggests that opioids can potentially activate TLR4 signaling, in the presence of MD2 or simply on their own, by binding MD2 and inducing TLR4 dimerization.

The most recent findings, pertaining to opioid analgesia, show that a series of novel (+)-Naltrexone-inspired analogs were able to antagonize TLR4 signaling. The lead opioid in this study was also tested *in vivo* in a Hargreaves assay, and was able to demonstrate efficacy as compared to a morphine/vehicle[30].

3. Agonists and antagonists of TLR4 with pharmaceutical potential

3.1. Designing TLR4 agonists and antagonists

Both antagonists and agonists of TLR4 were demonstrated to hold high pharmaceutical value, with indications in chronic neuralgia or opioid hyperalgesia with TLR4 activation (Table 3). Designing and screening of potential antagonist or agonist molecules benefits from recent studies on structural biology [10c, 24, 31]. The mechanism of TLR4 binding to its accessory proteins has been revealed *via* the crystal structure of the $(TLR4\text{-}MD2\text{-}LPS)_2$ dimer complex and the crystallographic data of MD2 binding to TLR4 antagonists (such as Eritoran) [28b, 32]. The most popular design strategy of antagonists utilizes the native ligand as inspiration for the antagonist molecule, which encourages competitive binding [10c, 33].

3.2. Formation of the LPS-MD2-TLR4 and Eritoran-MD2-TLR4 complexes

The natural TLR4 agonist, (LPS) and structurally related antagonist, (Eritoran), both contain an *N*-acetylglucosamine disaccharide scaffold with two phosphates in positions 1 and 4. One way in which these two molecules differ is that LPS contains six large lipid chains, where as Eritoran contains only four [10c]. The head portion of LPS, known as Lipid A, is able to fill a hydrophobic cavity formed on the MD2 protein with its six lipid chains. When all six lipid chains occupy the MD2 cavity, the disaccharide scaffold is situated just above the cavity, such that the two phosphate groups are able to interact with positively charged residues on both TLR4 molecules to induce dimerization [28a].

Further crystallographic studies have demonstrated that the size of the MD2-binding cavity is relatively unchanged when bound to Eritoran as compared to LPS. Since Eritoran has two fewer lipid chains, the MD2 binding cavity can easily accommodate the smaller ligand [28a]. Because of the smaller volume requirements of Eritoran, the ligand is able to settle further into the MD2 binding cavity as compared to LPS. As a result, the glucosamine backbone of

Eritoran is lowered closer to the rim of the cavity by approximately 5Å. This backbone shift allows the two phosphate groups to interact with positively charged residues on MD2, rather than with either of the TLR4 molecules, thus inhibiting TLR4 signaling [28a, 32].

3.3. Targeting MD2-TLR4 binding

3.3.1 Design of MD2-I—Peptide MD2-I was reported in 2009 as an antagonist of TLR4 by reproducing the TLR4-binding region of MD2 [12b]. By rationalizing a 17-residue peptide (CRGSDDDYSFCRALKGE) with a disulfide bridge between Cys95 and Cys105, MD2-I can mimic the critical TLR4-binding region of MD2, which is confirmed by circular dichroism (CD) experiments [34]. *In vivo* experiments using HEK293 cells suggest that MD2-I behaves similarly to the Cys95Tyr MD2 mutant, which proved the hypothesis that MD2-I might block TLR4 downstream proinflammatory effectors by blocking the TLR4-MD2 interaction [12b] (Figure 4).

3.3.2. Designs based on the MD2-I structure—Recent studies by Yin's group on MD2-I have proved that the key structure of MD2 in MD2-TLR4 binding is a short peptide segment with only 10 residues. With *Rosetta* software, contribution of this 10-residue segment in binding energy of TLR4 and MD2 interaction can be calculated accurately as 53% [35]. It is reasonable to predict that a more ridged version of this sequence could be a template of TLR4 agonists. This was achieved by the synthesis of a disulfide-bridged macrocycle By substituting an alanine (Ala) for a Cysteine (Cys) in YH1 and YH3, which prohibits disulfide bond formation, two linear-structured peptide YH2 and YH4 were synthesized as reference samples (Table 4). *In vivo* experiments demonstrated that YH1 and YH3 with disulfide-bridged structure show much stronger ability to synergistically activate TLR4 signaling in the presence of LPS than their linear counterparts YH2 and YH4 [36]. Detection of NO production in RAW264.7 cells also suggests that TLR4 activity may be related to the size of macrocycle formed by disulfide bond. The activity of TLR4 stimulated by YH3 is higher than YH1, which may suggest that larger rings might be less effective in binding TLR4 [36].

3.3.3. β-Amino alcohol derivatives—Besides MD2-based antagonists, other antagonists have been designed to block MD2-TLR4 binding. β-Amino alcohol derivatives have proved to be effective suppressors of TLR4 activation, and further experiments on clinical uses are ongoing [37]. Yin and co-researchers have discovered an innovative, robust method to synthesize β-amino alcohol derivatives, such as T5342126, **8**, which is a TLR4 antagonist (Table 5) [37a]. Besides T5342126, Yin's group also used this new synthetic method to prepare a focused library of analogs, and test their potential inhibitive effects on TLR4 activation in HEK293 cells by SEAP assay [12b, 38]. In this library, compound **10** was confirmed to be the most efficient TLR4 inhibitor. While compound **9** having little inhibition suggests that the functional groups on the aromatic rings (R1 and R2 in Table 5) might have great influence on the activity of these TLR4 antagonists [38]. We can only speculate that the electronic nature of these two rings play an important role in the binding of these molecules to TLR4, and must be fine-tuned to facilitate inhibition. Further results have demonstrated the ability of TLR inhibitors to enhance morphine's analgesic effect by blocking TLR4 activation [39]. Hargreaves test is a common method to test the intensity of a compound's

analgesic effect, by measuring the time taken to observe radiant heat-induced withdrawal response by hindpaws and tails of unrestrained rats after compound injection [40]. Both compound **8** and compound **10** have proven to be able to enhance morphine's analgesic effects, while no analgesic effect was detected when these compounds were injected alone [37a, 38].

Additional efforts based on **8** have recently been published [41]. An *in silico* screening was performed, favoring molecules with high similarity to **8**. These virtual screening results provided 45 potential hit molecules to be further evaluated. The field was further narrowed to fourteen compounds, by elimination of toxic and insoluble compounds. Three of the fourteen compounds (Figure 5) showed favorable IC_{50} values (ranging from 16.6 μM to 167.5 μM) in a SEAP assay utilizing HEK293 cells. Surface plasmon resonance (SPR) was used to further demonstrate binding of their lead compound to immobilized recombinant human TLR4.

3.3.4. Tanshinone IIA—Another small molecule that has been shown, in a recent study, to effectively modulate TLR4 activity is Tanshinone IIA (Tan IIA)[42]. Spinal nerve ligation (SNL), a popular model used to generate neuropathic pain *via* the ligation of the L5 and L6 spinal nerves [43], was performed on male Sprague-Dawley (SD) SPF rats. The rats were divided into three random groups post-operation: sham group, vehicle treated group, and Tan IIA treated group. Paw mechanical withdraw threshold (PWT) was used to measure mechanical allodynia, and paw thermal withdrawal latency (PWL) was used to measure thermal hyperalgesia. The Tan IIA treated rats displayed a higher threshold for pain in both the PWT and PWL tests as compared to the sham and vehicle groups. Furthermore, HMGB1 and TLR4 genes and protein were downregulated in the spinal cords of the Tan IIA treated group. Finally, the proinflammatory cytokines IL-1β, IL-10, and TNFα were also downregulated in the Tan IIA treated group. This study serves as an example that small molecules can effectively treat pain in mammals.

3.3.5. TAK-242—Perhaps the most advanced small molecule modulator of TLR4 is TAK-242 [44] (Figure 6). TAK-242 has been show to covalently bind to Cys747 of the TIR domain of TLR4, rendering TLR4 completely inactive. However, TAK-242 failed in phase 3 clinical trials in the treatment of sepsis because this inhibition failed to effectively reduce the levels of pro-inflammatory cytokines in humans with severe sepsis. The reason for this is unknown. One theory is that alternative pathways may be able to mediate the inflammation response without TLR4. This is supported by a recent study showing that caspase-11 and caspase-4 are capable of binding intracellular LPS, thus killing the cell [45]. These results, however, should not discourage researchers pursuing TLR4 inhibitors for neuropathic pain. A recent study demonstrated that naturally TLR4 deficient C3H/HeJ mice are less sensitive to stress-induced visceral pain than wild-type C3H/HeN mice [46]. The researchers further supported this finding by administering TAK-242 to mice with functional TLR4, and observing that the mice had acquired a higher threshold for visceral pain. Futhermore, TAK-242 was able to counteract chronic stress-induced visceral hypersensitivity. These results were not reproduced with the TLR4 deficient C3H/HeJ mice. As expected, the levels of pro-inflammatory cytokines IL-6 and TNFα were reduced after TAK-242 treatment. A

recent study, however, cautions that TLR4 inhibitors, such as TAK-242, can impair the endogenous opioid-mediated analgesia [47]. The researchers demonstrate this effect in Wistar rats with complete Freund's adjuvant (CFA)-induced paw inflammation. TAK-242 was shown to block the release of endogenous endorphins, which are normally released to provide natural analgesia.

3.4 LPS sequestrants

LPS sequestrants can block TLR4 activation by sequestering the LPS molecule [44]. Structural analysis of the LPS-binding domains of various proteins which have strong affinity for LPS (such as bactericidal/permeability-increasing protein (BPI), and Limulus anti-LPS factor (LALF), and LBP), reveals an LPS-binding motif which is functionally independent of LPS transport or neutralization [10c, 27, 48]. With the help of this structural motif, several peptides were discovered which could specifically bind to LPS on Gram-negative bacteria. These peptides consist of a long β-strand, which has alternating bulky and basic hydrophobic amino acids. Binding of these peptides to LPS suppresses TLR4 activation and TLR4 downstream cytokine production *in vitro* and in animal models [49]. Polymixin B (PMB) was an LPS sequestrant with this motif structure, whose therapeutic potential has been proved, and PMB has already been approved for clinical use in Japan [10c, 47, 50].

3.5. Blocking LPS-LBP or LPS-CD14 binding

Interfering with LPS-LBP or LPS-CD14 dimer formation could also block TLR4 activation. Structural analysis of the CD14 monomer shows a horseshoe-shaped structure containing a concave surface formed by a large β-sheet, which consists of leucine-rich repeats (LRR). Those LRRs result in the formation of several grooves and pockets, which are essential for LPS binding [51]. Based on this structure, a glycoconjugate obtained from Treponema spirochetes (Tm-Gp) has been demonstrated to be a potential TLR4 antagonist, by blocking LPS binding to CD14 and LBP (Figure 7) [52]. New artificially synthesized molecules, compounds **1**-**4** (Figure 6), were reported in 2009 as possible antagonists for TLR4 [53]. The inhibition of LPS- and lipid A-promoted cytokine production in macrophages and dendritic cells was observed during relevant experiments [10c, 53].

3.6. Tricyclic anti-depressants work on TLR4 with an opioid-like mechanism

Opioids compete with LPS for binding to MD2 [24]. Many morphine-like compounds are speculated to be potential TLR4 antagonists or agonists by the same mechanism. Hutchinson and co-researchers' studies have demonstrated the potential of tricyclic anti-depressants to be TLR4 antagonists [37b]. Tricyclic compounds have proved to be effective anti-depressant drugs for the nervous system, and they may share the same mechanism with opioids for activating TLR4 [45–46]. Eight tricyclic compounds were tested as potential antagonists or agonists of TLR4. Compounds were added into HEK293 cell cultures, either alone or with classical TLR4 agonists, to test their influence on TLR4 activation. Six compounds presented varying degrees of inhibition on TLR4 activation, including amitriptyline, imipramine, mianserin, cyclobenzaprine, ketotifen and desipramine (Figure 6). Other two compounds, carbamazepineand and oxcarbazepine, presented a mild agonistic effect on

TLR4 [37b]. Those two compounds induced more distinct TLR4 activity without LPS addition. Hutchinson's group speculated that tricyclic compounds share a similar mechanism with opioids on activating TLR4. They used AutoDock4, the latest version of the popular docking software, to confirm the binding of compounds docked to MD2 *in silico* [37b]. Most of the compounds were docked with a greater preference to the LPS binding cleft of MD2, instead of the TLR4 binding cleft. These docking results support the idea that tricyclic anti-depressants are competitive with LPS in their binding to MD2, just as the opioids are [37b]. Together with experimental *in vivo* results, Hutchinson's group built a new prediction model for both TLR4 activators and inhibitors. The prediction model is mainly based on experimental results of four tricyclic ligands (oxcarbazepine, carbamazepine, amitriptyline and mianserin). Agonism and antagonism of TLR4 by other potential small molecules are predicted under this new model in order to confirm their applicability. Compared to experimental data obtained *in vivo*, predicted results are very close to actual results, with most errors being less than 1% [37b]. Further experimental results *in vivo*, such as intracellular TLR4 signaling in RAW264.7 cells and TLR-induced interleukin-1 release in murine microglial BV-2 cells, also support those conclusions. [37b] (Figure 8)

3.7. Blocking CD14 binding

Peri's group mainly focuses on the accessory protein CD14. Haemin (Figure 6) is a common compound that can activate macrophages [53b]. Since haemin activation requires participation of CD14 and TLR4, it is speculated that haemin is a CD14 dependent TLR4 agonist [54]. Besides haemin, Peri's group also synthesized several sugar-derived small molecules, composed of a glucose unit linked to two C_{14} chains, and either nitrogen substituent or ether linkage on C-6, as potential CD14-dependent antagonists. While those three compounds showed obvious inhibition of TLR4 in both HEK293 cells and murine cells, another similar sugar-derived compound with a positive charge on C-6 has little inhibition [55]. Thus, the electronic properties of the C-6 nitrogen atom may be key to blocking CD14 binding [53b].

4. Prospective

The mechanistic differences between TLR4 agonists and TLR4 antagonists still remain unsolved. Before TLR4 modulating drugs can be introduced for neuroinflammation, further preclinical and clinical research must be developed. Another focus of TLR4-based drug development is decreasing the molecular weight of new drugs, especially analogs of LPS, which could improve drug absorption, and more effectively inhibit or stimulate TLR4 activity in the human body.

As highlighted in this review, the literature in the field of Toll-like receptors and neuropathic pain is continually growing. Researchers, armed with a growing list of TLR4 and MD2 based crystal structures, are finding more success with *in silico* docking simulations, which aids them in launching new projects. Additionally, more projects are yielding pre-clinical candidates with promising results from *in vivo* animal studies. With so much effort and early success in this ever-growing field, there should be applications for TLR4 modulators to treat pain in neurologic or analgesic clinics in near future.

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Abbreviations

TLRs	Toll-like receptors
DAMPs	danger associated molecular patterns
PAMPs	pathogen associated molecular patterns
MyD88	myeloid differentiation primary-response gene 88
TRIF	Toll/IL-1R (TIR)-domain-containing adaptor protein
TIRAP	TIR domain containing adaptor protein
MAL	MyD88 adaptor-like protein
TRAM	TRIF-related adaptor molecule
TRAF	tumor-necrosis-factor-receptor-associated factor
TBK1	TRAF-family-member-associated NF-κB activator (TANK)-binding kinase 1
RIP1	receptor-interacting protein 1
IRAK	IL-1R-associated kinase
TAK1	transforming-growth-factor-β-activated kinase
TAB	TAK1-binding protein
NF-κB	nuclear factor-κB
IKK	inhibitor of NF-κB kinase
IκB	inhibitor of NF-κB
TNF	tumor-necrosis factor
IRF	interferon regulatory factor
IFN	interferon
MKK	mitogen-activated protein kinase kinase
LPS	lipopolysaccharide
LBP	lipopolysaccharide binding protein
MD-2	myeloid differentiation protein 2
CNS	central nervous system

NO	nitric oxide
PRRs	pattern recognition receptors
CD14	cluster differentiation antigen 14
M3G	morphine-3-glucuronide
M6G	morphine-6-glucuronide
LRR	leucine-rich repeats
Tm-Gp	glycoconjugate preparation from Treponema spirochetes
Et	Ethyl group
BPI	bactericidal/permeability-increasing protein
LALF	Limulus anti-LPS factor
MAPK	mitogen-activated protein kinase
RelA	p65 subunit of NF-κB
PI3K	phosphoinositide-3-kinase
Akt (PKB)	protein kinase B

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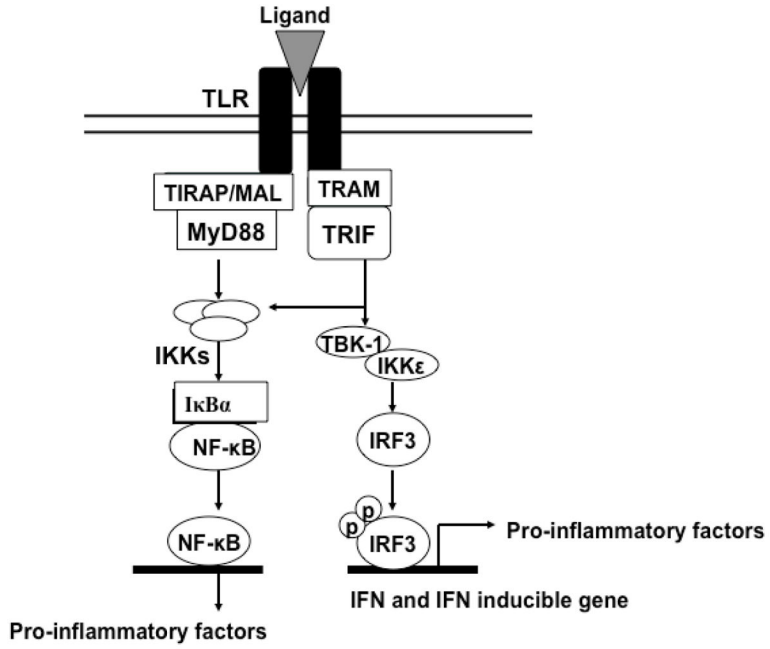


Figure 1.

Myeloid differentiation primary response gene 88 (MyD88) is the key signaling adaptor for TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, TLR8 and TLR9. Only TLR3 and TLR4 signal *via* TIR-domain-containing adapter-inducing interferon-β (TRIF). MAL, MyD88 adaptor-like protein; TRAM, TRIF-related adaptor molecule; TBK1, tumor necrosis factor receptor-associated factor (TRAF) family member-associated NF-κB activator (TANK)- binding kinase 1; IKK, inhibitor of NF-κB kinase; IκB, inhibitor of NF-κB; NF-κB, nuclear factor-κB; IRF, interferon regulatory factor; IFN, interferon.

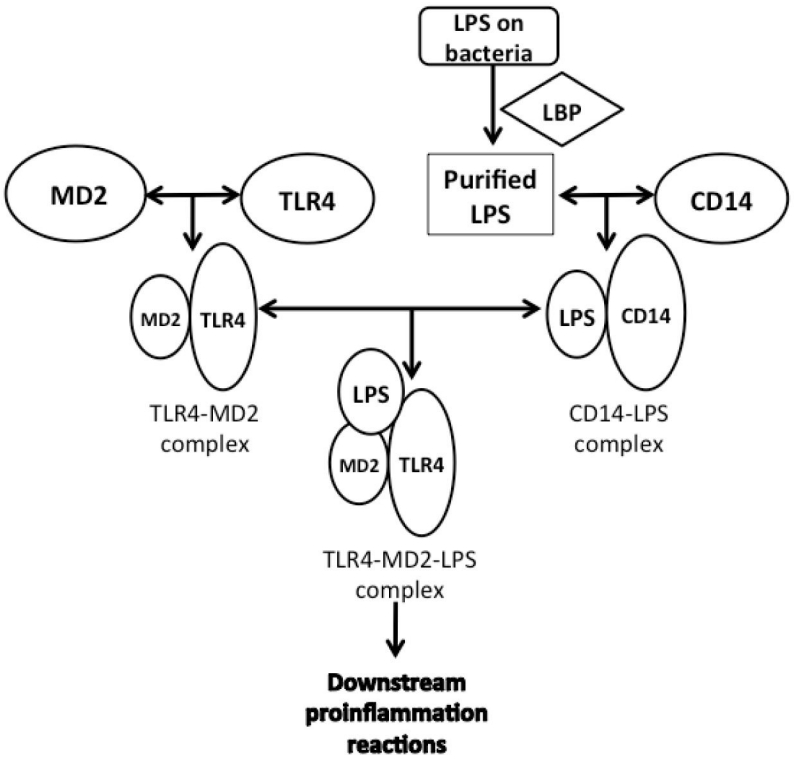


Figure 2.

TLR4 activation requires the participation of some accessory proteins besides TLR4 and LPS. LBP extracts LPS from bacteria and transports it to CD-14 to form a dimer (soluble $CD14\text{-}LPS)_2$ complex or monomer (membrane CD14-LPS). Either of the CD14-LPS complexs can bind to the MD2-TLR4 complex to form a ternary TLR2-MD2-LPS complex, which triggers downstream pro-inflammatory signaling.

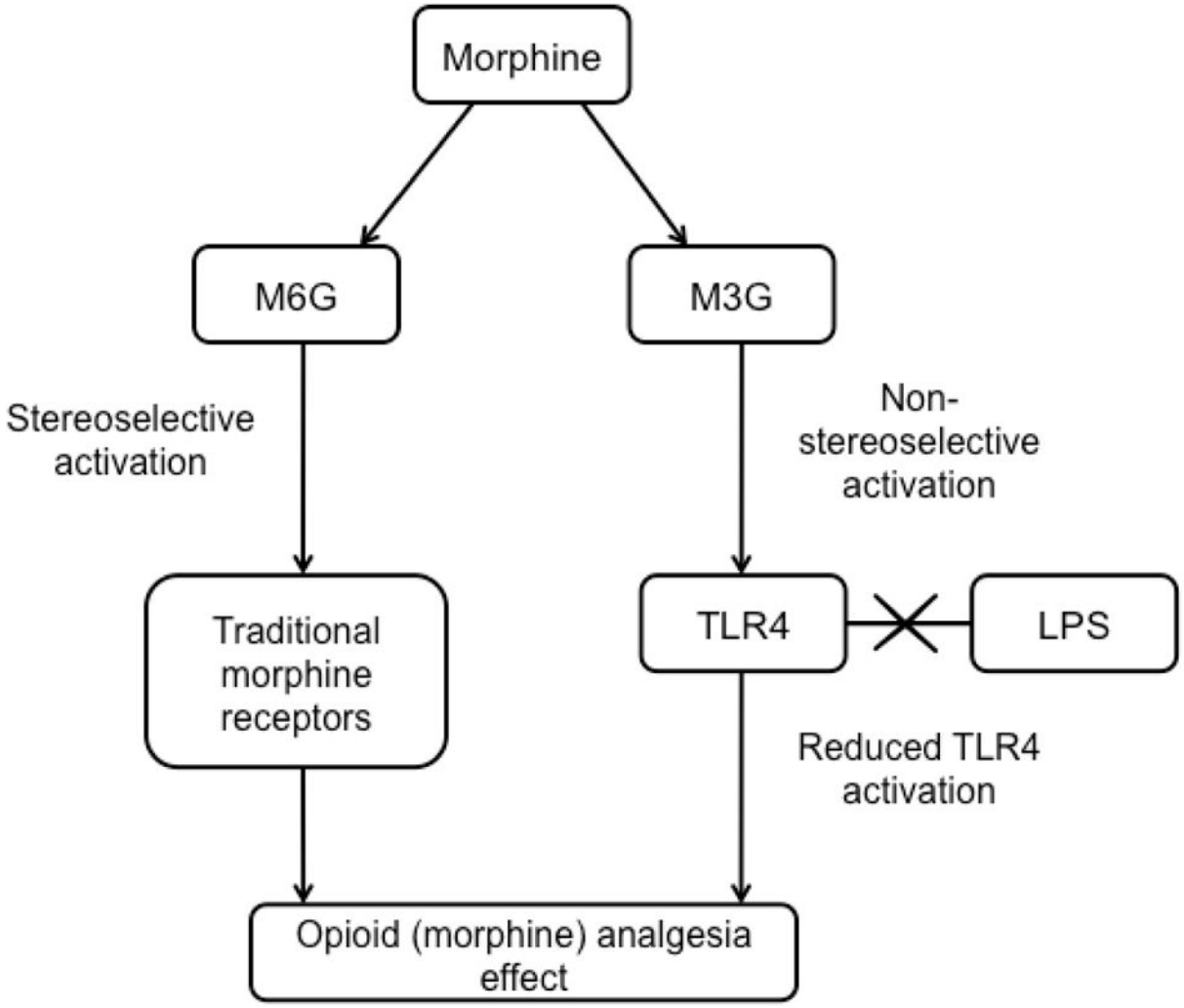


Figure 3.

Morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) are both morphine metabolites. In the stereoselective morphine activation, specific receptors of morphine bind with M6G. However, non-stereoselective morphine activation requires the participation of M3G, for TLR4 on glia cells. M3G competes with LPS for the same binding site on TLR4, which in turn results in the potentiation of the opioid analgesic effect.

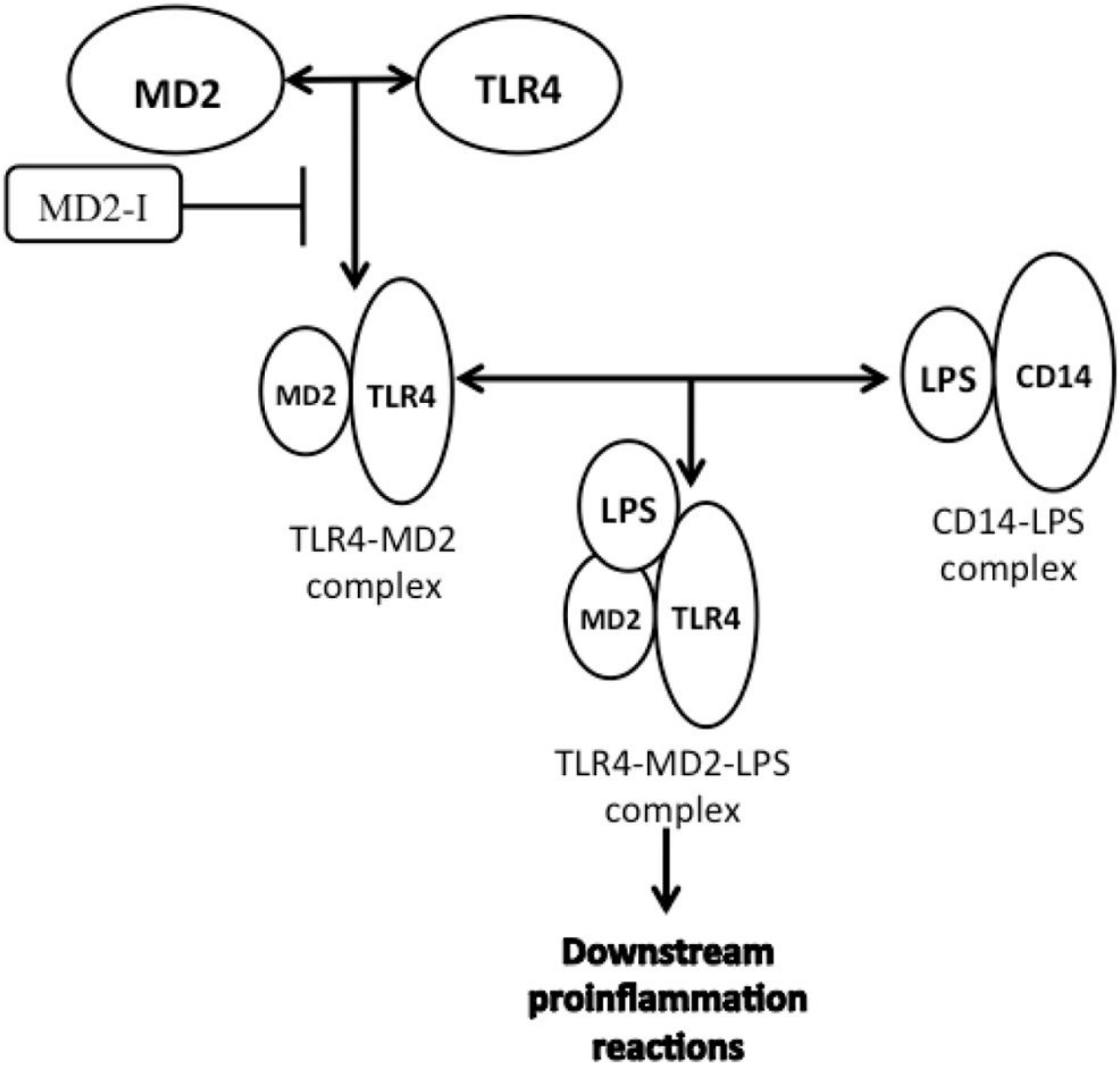


Figure 4.

Design of MD2-based TLR4 antagonists utilizes the structural similarity of agonists and MD2, which results in competition between antagonists and MD2 for TLR4 binding. For TLR4 binding with antagonists, such as MD2-I, the formation of the MD2-TLR4 complex may be blocked.

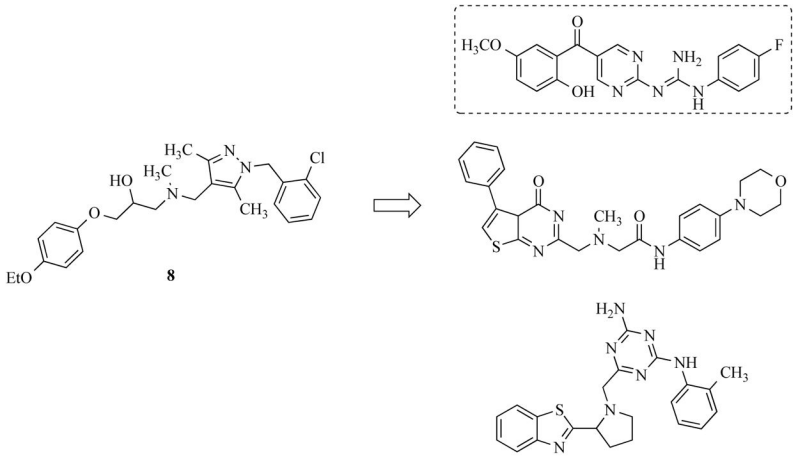


Figure 5.

A T5342126 (**8**,) inspired similarity search identified three new potential TLR4 signaling inhibitors *in silico*. The lead compounds (shown in box, with an IC_{50} of 16.6 μM) ability to bind TLR4 was confirmed in an SPR experiment with chip immobilized TLR4.

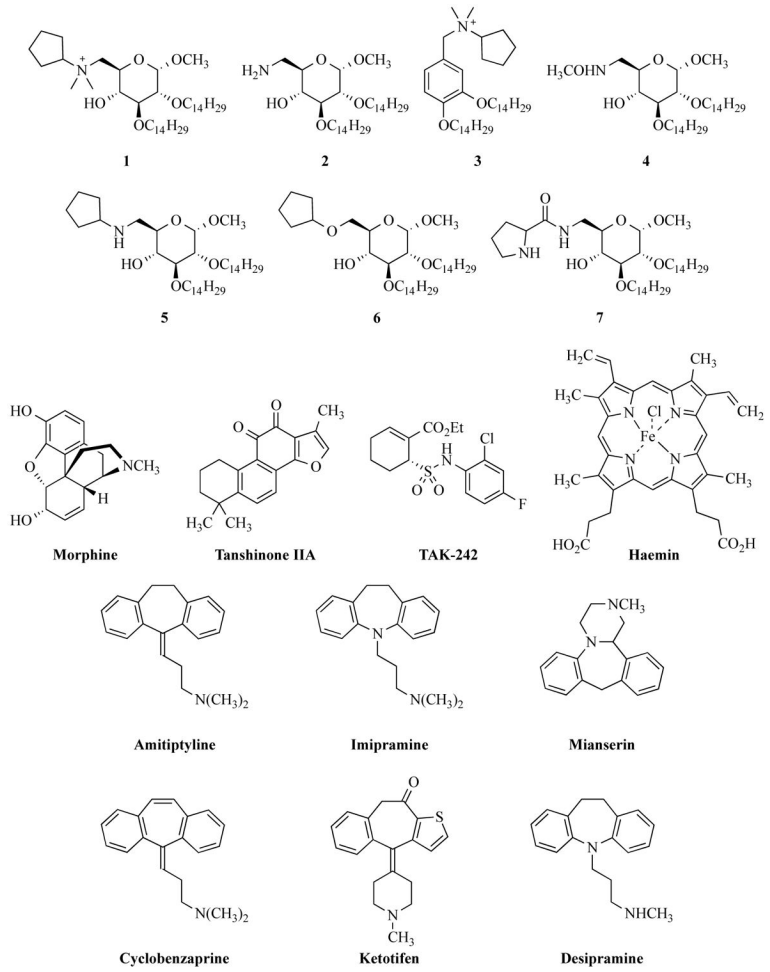


Figure 6.
Various small molecules which modulate TLR4 activity.

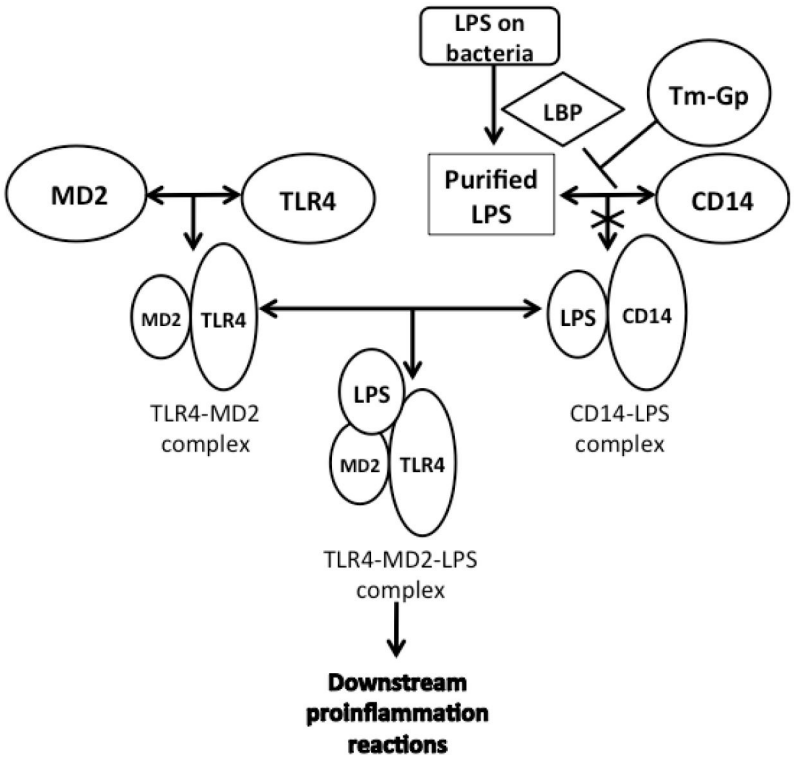


Figure 7.

A glycoconjugate obtained from Treponema spirochetes (Tm-Gp) has been demonstrated to be a potential TLR4 antagonist by blocking LPS binding to CD14 and LBP. Tm-Gp was designed based on the CD14 monomer structure, which has a horseshoe-shaped structure containing concave surface formed by a large β-sheet.

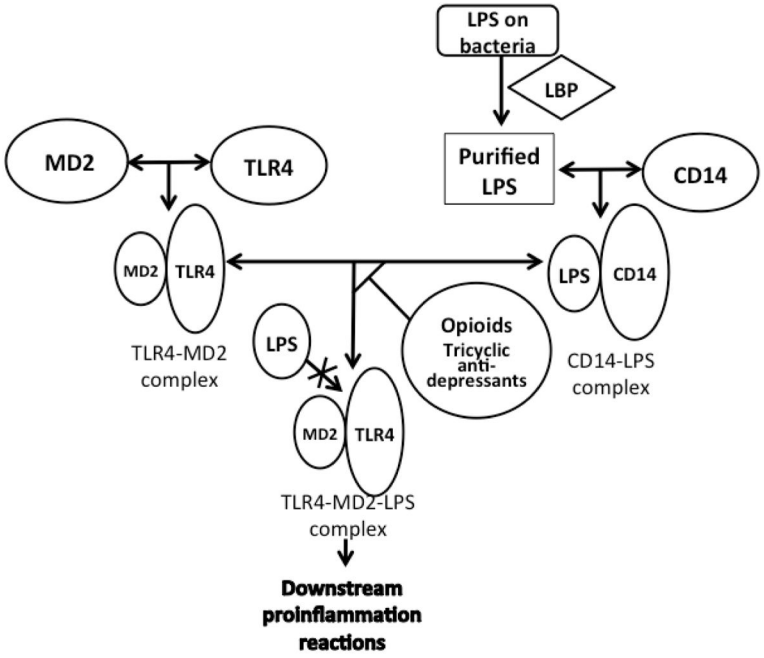


Figure 8.

Different from MD2-I based antagonists, opioid and opioid-like TLR4 antagonists compete with LPS for MD2 binding which reduces TLR4 activity.

Table 1

List of human and rodent TLRs

TLR	Exogenous ligands	Origin of ligands	References	Endogenous ligands	References	Localizations
TLR1	Triacyl lipopeptides	bacteria and mycobacteria	[56]			cell surfaces
	Soluble factors	*Neisseria meningitidis*				
TLR2	Lipoprotein/lipopeptides	various pathogens	[57]	Heat-shock protein 70	[7d]	cell surfaces
	Peptidoglycan	gram-positive bacteria	[58]	HGMB1	[59]	
	Lipoteichoic acid	gram-positive bacteria	[58b]			
	Lipoarabinomannan	mycobacteria	[60]			
	Phenol-soluble modulin	*Stapylococcus epidermidis*	[61]			
	Glycoinositolphospholipids	*Trypanosoma cruzi*	[62]			
	Glycolipids	*Treponema maltophilum*	[63]			
	Porins	*Neisseria*	[64]			
	Atypical lipopolysaccharide	*Leptospira interrogans*	[65]			
	Atypical lipopolysaccharide	*Porphyromanas gingivalis*	[66]			
	Zymosan	fungi	[67]			
TLR3	Double-stranded RNA	viruses	[68]	Double-stranded DNA	[69]	endosomes
TLR4	Lipopolysaccharide	gram-negative bacteria	[70]	Heat-shock protein 70	[71]	cell surfaces
	Fusion protein	respiratory syncytial virus	[72]	Type III repeat extra domain A of fibronectin	[73]	
	envelope protein	mouse mammary-tumor virus	[74]	Oligosaccharides of hyaluronic acid	[75]	
	Heat-shock protein 60	*Chlamydia pneumoniae*	[76]	Polysaccharide fragment of heparan sulphate	[77]	
				Fibrinogen	[78]	
TLR5	Flagellin	bacteria	[79]			cell surfaces
TLR6	Diacyl lipopeptides	Mycoplasma	[80]			cell surfaces
	Lipoteichoic acid	gram-negative bacteria	[58b]			
	Zymosan	fungi	[81]			
TLR7	Single-stranded RNA	viruses	[82]	Single-stranded RNA	[83]	endosomes
	Imiquimod (R837)	synthetic compound (FDA approved drug)	[84]			
TLR8	Single-stranded RNA	viruses	[82a]	Single-stranded RNA	[83]	endosomes
TLR9	CpG-containing DNA	bacteria and viruses	[85]	DNA	[86]	endosomes

TLR	Exogenous ligands	Origin of ligands	References	Endogenous ligands	References	Localizations
TLR10	Not Determined	Not Determined				Not Determined
TLR11	profilin	Uropathogenic pathogen, toxoplasma	[87]			Not Determined
TLR13	23s rRNA	gram-negative/positive bacteria	[88]			Not Determined

Table 2

The expression of TLR mRNA and TLRs in different human neural cells

Neural Cells	TLR mRNA	TLRs Protein	References
Microglia	TLR1-9	TLR1-4	[18a, 18c, 89]
Astrocytes	TLR1-5, TLR9	TLR3, TLR4	[18a, 90]
Oligodendrocytes	TLR1-9	TLR2	[89, 91]
Schwann cells		TLR2	[18e]
Neurons	TLR1-4	TLR3, TLR4, TLR7, TLR9	[92]

Table 3

TLR4 antagonists and agonists

Mechanism	Antagonist/agonist	References
Block binding between MD2 and TLR4	MD2 I	[12b]
	YH1/YH3	[36]
	β-amino alcohol derivatives	[37a]
Block binding between MD2 and LPS	Tricyclic anti-depressants	[37b]
LPS sequestrants	Polymixin B	[18g, 40, 47]
Block binding of CD-14	Haemin and three sugar-derived small molecules	[54] [55]

Table 4

Peptide sequences and chemical formulas of synthesized TLR4 agonists, antagonists, and analogs

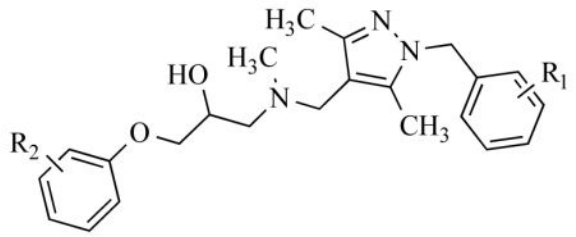
Peptides	Amino acid sequences
MD2-I	Disulfide bond ***C***RGSDDDYSF***C***RALKGE
YH1	D100C mutation to form disulfide bond D***C***DYSFCR***A***L
YH2	No disulfide bond D***A***DYSFAR***A***L
YH3	Disulfide bond forms a bigger macrocycle ***C***AADDDYSFCRALAA***C***
YH4	No disulfide bond ***A***AADDDYSFCRALAA***A***

Bold and italic styles are used on residues that are supposed to form disulfide bonds.

Underlined font indicates the mutated residues.

Table 5

Results from the SEAP Reporter Gene Activation Assay*



Compound	R1†	R2†	Inhibition (%)
8	2-Cl	4-OEt#	52
9	H	H	1
10	2-Cl	4-Cl	99

*The percent of inhibition was determined by measuring LPS-induced TLR4 activation in HEK293 cells, in the presence of 50 μM drug (compound) as compared to blank control.

#Et = Ethyl group.

†R1 & R2 = functional groups on the aromatic rings of T5342126-based inhibitors.