Toll-like Receptor 4 (TLR4) modulation by synthetic and natural compounds: an update

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Abstract

Toll-like receptor 4 (TLR4), together with MD-2, binds bacterial endotoxins (E) with high affinity, triggering formation of the activated homodimer $(E\text{-}MD\text{-}2\text{-}TLR4)_2$. Activated TLR4 induces intracellular signaling leading to activation of transcription factors that result in cytokine and chemokine production and initiation of inflammatory and immune responses. TLR4 also responds to endogenous ligands called danger associated molecular patterns (DAMPs). Increased sensitivity to infection and a variety of immune pathologies have been associated with either too little or too much TLR4 activation. We review here the molecular mechanisms of TLR4 activation (agonism) or inhibition (antagonism) by small organic molecules of both natural and synthetic origin. The role of co-receptors MD-2 and CD14 in the TLR4 modulation process is also discussed. Recent achievements in the field of chemical TLR4 modulation are reviewed, with special focus on non-classical TLR4 ligands with a chemical structure different from lipid A.

Introduction

Toll-like receptors (TLRs) play a critical role in the recognition of conserved pathogen-associated molecular patterns (PAMPs) derived from various microbial pathogens including viruses, bacteria, protozoa and fungi, and in the subsequent initiation of innate immune responses.[1] Among TLRs, TLR4 selectively responds to bacterial endotoxin (E), Gram-negative bacterial lipopolysaccharides (LPS) or lipooligosaccharides (LOS).[2,3] In addition, TLR4 recognizes a broad variety of substances from viruses, fungi, and mycoplasma.[4] TLR4 is also activated by endogenous factors, generally known as danger (or damage) associated molecular patterns (DAMPs).[5] Typical DAMPs acting as TLR4 agonists are endogenous substances which are released as a consequence of injury and inflammation. They include β-defensin, high-mobility group protein 1 (HMGB1), heat shock proteins (HSP), hyaluronic acid, heparin sulfate, substance P, and others. It remains a challenge to provide unequivocal evidence that DAMP proteins are direct TLR4 ligands, as the DAMP proteins used in experiments are often contaminated with endotoxin and other TLR ligands introduced during protein expression and/or purification. Targeting activation of TLR4 by bacterial endotoxin (LPS) is important in order to develop drugs active against acute sepsis and septic shock derived from excessive and deregulated TLR4 activation and signaling.[6] The inhibition of TLR4 stimulation by endogenous factors could be used to contrast a wide range

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of inflammatory and autoimmune disorders associated to the release of Reactive Oxygen or Nitrogen Species (ROS/RNS) and inflammatory cytokines following "sterile inflammations" induced by DAMPs. A recent review on TLR literature emphasizes TLR4 as an emerging molecular target related to an impressively broad spectrum of modern day disorders[6] including asthma, cardiovascular disorder, diabetes, obesity, metabolic syndrome, autoimmune disorders, neuroinflammatory disorders, neuropathic pain, CNS disorders such as amyotrophic lateral sclerosis (ALS) and Alzheimer Disease (AD), psychiatric diseases, skin inflammations (dermatitis), psoriasis and some tumors. As the majority of these pathologies still lack specific pharmacological treatment, small molecules active in inhibiting TLR4 activation have attracted increasing interest in a wide range of possible clinical settings.

The TLR4 activation process

The mechanism by which LPS activates TLR4[7] is the one we understand best. Activation of TLR4 by LPS is a complex process and depends on LPS binding protein (LBP)[8]-catalyzed extraction and transfer of individual LPS molecules from aggregated LPS to cluster of differentiation 14 (CD14),[9] and then from CD14 to myeloid differentiation protein 2 (MD-2),[10] followed by engagement and dimerization of TLR4 thus forming the activated complex (LPS.MD-2.TLR4)$_2$.[11] Crystal structures of the human[11] and murine[12] (LPS.MD-2.TLR4)$_2$ complex reveal that five of six acyl chains of lipid A moiety of LPS insert into the hydrophobic binding pocket of the MD-2 bound to TLR4. It is clear from X-ray structures that the sixth acyl chain stays on the rim of the cavity, and binds to a second TLR4 molecule that is also part of an LPS.MD-2.TLR4 complex.[11,12] However, recently published NMR studies indicate that protrusion of one of the six fatty acyl chains of endotoxin bound to MD-2 precedes interaction with TLR4 when endotoxin is bound to MD-2. According to NMR data, the presence of a protruding fatty acyl chain is not necessarily a distinguishing feature of TLR4-activating LPS.MD-2 complexes and so is not sufficient for driving TLR4 activation.[13] The intracellular signal triggered by TLR4 dimerization consists of two different pathways, the myeloid differentiating primary response gene 88 (MyD88)-dependent and the MyD88-independent pathway based on the TRIF and TRAM effectors. TLR4 is unique among the TLRs in triggering two distinct signal/transduction pathways.

While the structural data of endotoxin-receptor complexes allowed the rational design of synthetic molecules able to compete with endotoxin (lipid A and LPS) for the binding to TLR4.MD-2 complex, very little is still known about the molecular details of the interaction between TLR4 and different DAMPs. The high chemical diversity of DAMPs structures probably reflects different modes of interaction with TLR4 and/or TLR4.MD-2 heterodimer. The role of accessory proteins CD14 and MD-2 is still controversial in the case of DAMP stimulation, and it has recently been proposed that, while CD14 might be a universal adaptor for PAMPs and DAMPs, MD-2 would selectively recognize only exogenous PAMPs.[14]

Structure-activity relationship in lipid A variants: from agonism to antagonism

The membrane-anchoring moiety of LPS, lipid A, is also the biologically active (toxic) part of the entire LPS and is composed of a glucosamine disaccharide core with two negatively charged phosphate esters at C1 and C4' positions and six fatty acid (FA) acyl chains linked to C2, C3, C2' and C3' disaccharide positions. While lipid A from *E. coli* (Figure 1) is considered the typical chemical structure associated to endotoxic properties, a variety of natural lipid A variants exist with modifications in the number and disposition of FA chains and with other covalent modifications that impact on pathogenesis, bacterial physiology and bacterial interactions with the host immune system.15

The chemical modification of the lipid A structure is now a well-established approach to TLR4 active molecules (Figure 1). Modifications of fatty acid (FA) acyl chains of lipid A, namely the removal of two or more FA chains, give the so-called underacylated lipid A variants containing less than six acyl chains. These natural or synthetic lipid A variants can block or inhibit the TLR4 activation, thus acting as antagonists (Figure 1). The crystal structure of TLR4.MD-2 homodimer bound to the tetracylated antagonist Eritoran16 revealed that Eritoran inserts all FA chains into the hydrophobic pocket of MD-2; however, in contrast to lipid A, Eritoran fails to induce TLR4 dimerization and activation, thus acting as a TLR4 antagonist. Interestingly, the tetraacylated lipid IVa, a biosynthetic precursor of lipid A, acts as antagonist on human but as an agonist on mouse TLR4. When bound to mouse MD-2 (mMD-2), lipid IVa presents one lipid chain on the surface of MD-2, which reveals striking similarity to the crystal structure of the agonist hexaacylated with human MD-2 (hMD-2) in the activated complex (lipid A.hMD-2.TLR4)$_2$.[12] This species specific activity of lipid IVa is attributed, among other factors, to the dissimilarities in the shape of the hydrophobic binding pocket of h- and mMD-2 and to the variations in the electrostatic potentials at the rim of the binding cavity of MD-2 and at the dimerization interface.12

Lipid A dephosphorylation or replacement of the reducing-end sugar with an aminoacid allowed non toxic TLR4 stimulants to be obtained, with agonist action. Synthetic low-toxicity TLR4 agonists monophosphoryl lipid As (MPLA) and aminoalkyl glucosaminide 4-phosphates (AGPs) are in use as vaccine adjuvants and in cancer immunotherapy (Figure 1). The low toxicity and adjuvant function is associated with a TRIF-selective signaling instead of MyD88-dependent stimulation for both AGPs[17] and MPLAs.[18]

Other TLR4 agonists and antagonists are shown in Figure 1. They are obtained by lipid A structure simplification, including monosaccharides (GLA and ONO compounds), and mimetics with a linear backbone (ER112022). These compounds have been reviewed elsewere.[19,20]

The TLR4 agonists and antagonists depicted in Figure 1, with a structure derived from *E. coli* lipid A, are amphiphilic molecules with a polar (charged) head linked to a variable number of lipophilic chains. The amphiphilic character results in the formation of micelles in an aqueous environment above their critical micellar concentration (CMC) that is normally very low (nM range). As a consequence, aggregated forms of synthetic lipid A

mimetic, and of natural lipid A and LPS, should predominate in the concentration range relevant for biological responses and for biochemical characterization in vitro. Accordingly, variables in the aggregation state and in the 3D form of endotoxin or synthetic molecule aggregates may directly influence the kinetics and potency of TLR4 activation and signaling.21 Differences in lipid A/LPS molecular structure may therefore influence the kinetics and potency of TLR4 activation by affecting the 3-D structure of aggregates of these molecules to which LBP, albumin, and CD14 must first interact to yield the monomeric endotoxin. protein (CD14; albumin) complexes that provide endotoxin monomer to MD-2 and MD-2/TLR4. Moreover, aggregates structure influences the geometry of monomeric endotoxin.MD-2.TLR4 complexes that determine TLR4 agonist or antagonist function.

While the aggregates conformation in solution has been related to bioactivity in the case of natural TLR4 ligands, very few studies have been done so far with synthetic ligands. Another important consequence of the amphiphilic character of these molecules is their low water solubility which sometimes complicates their handling *in vitro* and *in vivo* and it is associated to poor distribution (pharmacokinetic) properties. This Mini-Perspective article presents the most recent achievements in the development of TLR4-active synthetic and natural small-molecules,19, 20 focusing on the mechanism of action and on the interaction with specific targets (TLR4, MD-2, and CD14 receptors). The extreme variety of chemical structures of small molecule TLR4-active compounds, especially in the case of natural compounds, is intriguing (Figure 2). As a consequence, these compounds target TLR4 and/or MD-2 and CD14 differently. However, the final common effect is the inhibition of the formation of the activated endotoxin.MD-2.TLR4 homodimer. Development of new molecular structures inspired by TLR4-active natural compounds, with improved water solubility, bioavailability and reduced toxicity compared to lipid A analogues, is one of the most promising future directions in this field, as will be discussed in this Perspective article.

Synthetic TLR4 modulators

Great synthetic effort has been produced through the years to explore the structure-activity relationship of different lipid A analogues and mimetics. Several TLR4-active compounds have been synthesized varying the nature, the position and the length of FA acyl chains and the number of phosphate groups, or using phosphate bioisoteres. Eritoran (Figure 1), with four acyl chains of different length and nature, is representative of this class of compounds. The rationale of antagonism of Eritoran relies on the capacity of this molecule to insert into the hydrophobic MD-2 binding cavity and occupy MD-2 without promoting the formation of TLR4.MD-2 heterodimer. Antagonist activity is shared by other underacylated lipid A variants with four fatty acid (FA) chains, as the biosynthetic precursor lipid IVa.[22]

Triacylated lipid A mimetics constituted by a diglucosamine core (Figure 3, compounds OM-174 type), or a pseudodipeptide backbone (compound **2**, OM-197-MP-AC,[23] Figure 3) with one or two phosphates have been recently synthesized. These compounds turned out to be active in modulating the activity of both TLR2 and TLR4. Diphosphorylated compound OM-174-DP[23] (Figure 3) showed antagonist action on TLR4 in HEK-BlueTM cells. The NF-κB activation in response to TLR4 stimulation can be measured in HEK-BlueTM

hTLR4 cells, which are stably transfected with human TLR4, CD14, MD-2 and a secreted embryonic alkaline phosphatase (SEAP) reporter gene. TLR4 antagonism was accompanied by an agonist effect on TLR2 with the level of activation of TLR2-expressing cells comparable to that observed with the classical TLR2 ligand Pam_3CSK_4. The change of anomeric substituents of compounds **1** (OM-174-MP-PD) and OM-174-MP-EP[23] (Figure 3) led to a significant lower activity as TLR4 antagonists. Otherwise compound **2** (Figure 3) showed an agonistic activity on TLR4, much lower than *E. coli* LPS, combined to a low endotoxic activity, thus representing a promising new vaccines adjuvant.

Compound **3**,[24] (D1, Figure 3), with four ether chains, is a non-classical lipid A mimetic in which two sulfate groups are linked to a core containing two α-D-glucopyranoside units bridged through a (C6-C6') succinic diamide linker. The sulfate groups, in anionic form at neutral pH, are bioisosteres of lipid A phosphates. In contrast to natural lipid A, **3** is symmetric with two-fold rotational symmetry (C2), and four linear ether chains ($C_{14}H_{29}$) replace the acyl esters ($COC_{13}H_{27}$ or $COC_{11}H_{23}$) found in *E. coli* lipid A and other synthetic antagonists. **3** inhibited in a dose-dependent manner the LPS-stimulated TLR4 activation in stable transformants of HEK293 cells expressing human TLR4 (HEK-TLR4 cells) supplemented with soluble MD-2, LBP and CD14.[24] **3** suppressed the formation of the $(LPS.MD-2.TLR4)_2$ complex *in vitro* and inhibited the transfer of tritiated LOS monomers from a preformed LOS-CD14 complex to MD-2, thus suggesting a high affinity binding of the synthetic compound with both CD14 and MD-2.

Lipid A mimetics in which the reducing-end glucosamine is replaced by an acylated diethanolamine moiety have been synthesized and showed a dose-dependent immunostimulatory activity in the human monocytic cell line THP-1.[25] Depending on the amine substituent, the molecules showed different potency in stimulating cytokines production, with compound **6** (Figure 3) containing a carboxylic acid group as a phosphate analogue exhibiting the greatest activity. A competitive inhibition study with lipid IVa gave indirect evidence that these compounds are TLR4 ligands, suggesting an interaction with the TLR4.MD-2 complex similar to that of the natural antagonist lipid IVa.[25]

In a further simplification of lipid A structure, different series of monosaccharidic glycolipids were found to activate or inhibit LPS-TLR4 signaling. Positively charged glycolipids with two C_{14} alkyl chains and a protonatable amine group on position C-6 of a methyl-α-D-glucopyranoside (IAXO 101 and IAXO 102, Figure 3) and aromatic compound IAXO 103 (Figure 3), inhibited TLR4 signaling *in vitro*[26] and suppressed some TLR4-related pathologies such as acute sepsis[26] and neuropathic pain[27] in mice. Experiments with purified receptors and NMR binding studies showed that IAXO compounds bind CD14 with higher affinity than MD-2.[28] Despite the striking structural difference with negatively charged lipid A, cationic IAXO compounds are active as TLR4 antagonists.

Synthetic serine-based analogues of α-galactosyl ceramide, in particular compounds **7**[29] (CCL-34, Figure 3), stimulated TLR4-dependent TNF-α production, while the natural α-galactosyl ceramide turned out to be inactive. Some variants of this molecule have been recently synthesized, to unravel the relationship between their chemical structure and immunostimulating properties. While the anomeric α-configuration is essential to the

biological activity, the galactose moiety can be replaced by other monosaccharides (α-fucose, α- or β-glucose, α-galacturonic acid) retaining the TLR4-stimulating activity, as assessed by experiments on HEK cells stably transfected with TLR4, MD-2 and CD14 receptors.[30] The antitumor potential of TLR4 agonist **7** was studied: macrophages activated by the synthetic ceramide induced cancer cell death via the apoptotic pathway and treatment with **7** suppressed tumor growth and increased the survival rate in TLR4-functional C3H/HeN mice but not in TLR4-defective C3H/HeJ mice.[31] Other natural and synthetic α-GalCer are known to possess immunostimulant activities, molecule KRN7000[32] (Figure 3), that is a simplified analogue of glycosphingolipids of marine origin is capable of activating natural killer T (NKT) cells. In the light of the recently observed TLR4 activity of **7**, the previously reported immunostimulating activity of some natural and synthetic α-GalCer is very likely due to TLR4 activation.

An alternative strategy to target TLR4 is to design low molecular weight inhibitors with a chemical structure totally different from lipid A, as in the case of compound **8**[33] (TAK-242, Figure 3) that, as Eritoran, reached phase III clinical trials as an antisepsis agent. **8** selectively inhibits TLR4 signal by covalently binding Cys747 in the intracellular domain of TLR4 and probably reacting with Cys thiol as a Michael acceptor, thus forming a covalent adduct with TLR4.[34] Yin and coworkers recently identified some β-aminoalcohol derivatives (Figure 3), as virtual candidates to disrupt the MD-2-TLR4 interaction. A panel of racemic and optically pure compounds derived from this scaffold inhibited TLR4-dependent nitric oxide (NO) production in RAW 264.7 macrophages and cytokine production in human whole blood with μM potency.[35] These compounds showed no cytotoxic activity at concentrations up to 300 μM, and the easily modified and scalable synthesis could provide more potent antagonists, studying a different arrangement of the aromatic substituents.[36]

In the course of an high-throughput screening designed to identify activators of innate immunity, a series of pyrimido[5,4-b]indoles,[37] with a structure totally unrelated to lipid A, were recently discovered as selective TLR4 activators (agonists). These agonists were active in stimulating the TLR4 signal in HEK293 reporter cell lines, and inactive on HEK cells expressing other TLRs. Interestingly, the activities of these compounds seem to be CD14-independent, as assessed by the capacity to stimulate cytokine production also in CD14-defective macrophages.[37] The direct binding to MD-2 of one of these compounds has been simulated by docking studies. Peptides are structurally versatile compounds that can be designed to interact in a complementary way with protein targets. Short peptides have been synthesized corresponding to the minimal TLR4-binding region of MD-2, and it was reported that they act as antagonists, inhibiting LPSinduced TLR4-dependent expression of proinflammatory cytokines both in human and in murine cells.[38] Peptide-based TLR4 agonists have also been developed recently, by screening a 7-mer phage-display peptide library.[39] The activity of the peptides in activating TLR4 signaling was assessed by NF-κB nuclear translocation analyses in HEK-blue™ cells. Furthermore, the peptides were capable of inducing inflammatory cytokine secretion from RAW264.7 cells.

Natural TRL4 modulators

Plant secondary metabolism provides an endless source of chemically diverse bioactive and pharmacologically active compounds. Plant-based extracts present a large source of natural immune modulators, many of which have been used in traditional medicine for centuries. Recent research efforts have identified plant extracts as potential modulators of TLRs.[40] The understanding of the molecular mechanisms of TLR4 activation promoted the study of TLR4 activity of some plant and herbal extracts with focus on the molecular mechanism of action of active principles.[4] Interestingly, many herbs used in Traditional Chinese medicine (TCM) and Ayurvedic medicine seem to be rich in molecules that interfere with TLR4 activation and signaling. These include green tea, *Glycyrrhiza uralensis*, better known as licorice, *Magnolia officinalis*, ginger (*Zingiber officinalis*), *Salvia miltiorrhiza* (red sage), and curcumin. The chemical structures of natural compounds whose TLR4 activity has been recently investigated are depicted in Figure 4. With some important exceptions, such as paclitaxel, morphine, opioids and sulforaphane, the TLR4-active compounds depicted in Figure 4 are polyphenolic and aromatic compounds sharing interesting similarities in their chemical structures and in their mechanism of action.

In particular, cinnamaldehyde, 1-dehydro-10-gingerdione (1D10G), 6-shogaol, isoliquiritigenin, curcumin and caffeic acid ester (Figure 4) contain α,β-unsaturated carbonyl moieties that can easily react as carbon electrophile with the nucleophilic thiol of a cysteine, thus forming Michael adducts with protein targets. The disruption of TLR4.MD-2 heterodimer by formation of covalent adducts with solvent-exposed MD-2 and/or TLR4 cysteines has been proposed as the main mechanism of action of **13**[41] (isoliquiritigenin), 6-shogaol,[42] caffeic acid phenetyl ester **14** (CAPE)[43] and cinnamaldehyde.[44] Despite the presence of the same α, β-unsaturated carbonyl functionality, curcumin does not seem to form a covalent bond with MD-2,[45] in contrast with a previously suggested mechanism of action.[46] Also compound **9** (1D10G) seems to inhibit the formation of TLR4 activated complex by interacting non-covalently with MD-2 and competing with endotoxin for MD-2 binding.[47] Sulforaphane contains a different electrophilic group, the isothiocyanato moiety, that forms covalent adducts with MD-2 exposed cysteine thiols, as recently observed by mass analysis.[48] The properties of the above-mentioned natural compounds on TLR4 signaling have been characterized through experiments on purified receptors, the molecular details will be discussed separately for each compound.

The molecular mechanism explaining the anti-inflammatory action of cinnamaldehyde (the major constituent of cinnamon bark from *Cinnamomum* trees, Figure 4), has recently been discovered.[44] It has been observed that cinnamaldehyde is active in inhibiting the activation of NFκB and IRF3 induced by LPS while being inactive in inhibiting NF-κB activation by downstream effectors such as MyD88 and IKKβ, thus suggesting an upstream effect on the extracellular TLR4.MD-2 complex. Furthermore, cinnamaldehyde inhibited TLR4 oligomerization induced by LPS in co-immunoprecipitation experiments. The observation that the saturated analogue of cinnamaldehyde, the 2-phenylpropionaldehyde, was inactive in inhibiting LPS-induced signaling, and the reactivity of cinnamaldehyde with other cysteine-exposing proteins suggested to the authors the formation of a covalent adduct cinnamaldehyde/MD-2. However, this adduct was not directly observed.[44] Curcumin

(Figure 4) is a polyphenolic compound isolated from the rhizomes of the plant *Curcuma longa* (turmeric) that has profound anti-inflammatory activity and is used in the treatment of sprain and swelling caused by injury in traditional Indian and Chinese medicine. It has been shown that curcumin inhibits not only the IκB kinase (IKK)β in the MyD88 signaling pathway but also the MyD88-independent pathway upstream of Toll/IL-1R domain-containing adaptor-inducing IFN-β(TRIF).[46] The binding between curcumin **10** and purified human MD-2 was investigated experimentally.[45] Fluorescence of **10** increased significantly upon addition of MD-2 and **10** inhibited in a dose-dependent manner the interaction between immobilized LPS and MD-2 in an ELISA-type assay, thus suggesting that LPS and **10** very likely compete for the same MD-2 binding pocket.[45] In this study authors also observe that **10** does not form a covalent linkage with MD-2 (in particular with Cys133 exposed on the inner surface of the MD-2 hydrophobic pocket), because denaturation of MD-2 subsequent to the formation of complex with **10**, followed by organic solvent extraction caused the complete transfer of **10** into the organic phase, demonstrating the noncovalent nature of the interaction with MD-2.[45]

Sulforaphane **11** (SFN, 1-isothiocyanato-4-(methylsulfinyl)-butane, Figure 4) is an organosulfur compound obtained from cruciferous vegetables such as broccoli or cabbages. Several studies documented that the well-established anti-inflammatory activity of **11** depends on suppression of TLR4 signaling. **11** attenuates LPS-induced nitric oxide synthase (iNOS), cyclooxigenase-2 (COX-2) and tumor necrosis factor-α (TNF-α) production in macrophage cell lines,[48] and inhibits NFκB translocation and IκBα degradation in LPS-stimulated endothelial cells and suppress LPS-induced expression of inflammatory genes.[48] Recently, it has been observed that **11** preferentially forms adducts with Cys133 in the hydrophobic pocket of MD-2, blocking the interaction with LPS and lipid IVa.[48] In Ba/F3 cells expressing TLR4 and Flag-tagged MD-2, treated with biotinylated LPS, **11** was observed to inhibit the association of LPS with MD-2 in a dose-dependent manner, as determined by immunoblot analysis. LC-MS/MS analysis after incubation of recombinant MD-2 with SFN showed the SFN covalently bound to Cys133 which is a free cysteine residue, but not to Cys95 and Cys105 which are engaged in the formation of intramolecular disulfide bonds.[48]

Ginger, the rhizome of the herb *Zingiber officinalis* has been traditionally used in Ayurvedic medicine to treat several inflammatory diseases and ginger extracts have anti-inflammatory and anti-cancer effects. The major molecular components that account for ginger's anti-inflammatory effects are gingerols and their dehydratation products, shogaols. The activity on TLR4 of some of these components, namely **9** (Figure 4), 6-, and 10-gingerdione, 4-, 6- and 10-shogaol (Figure 4) and gingerenone A was recently compared.[47] **9** is the most potent inhibitor of LPS-MD-2 interaction as determined by an ELISA test, the 6-gingerol and 10-shogaol were also active in inhibiting the interaction, while, surprisingly, the 6-shogaol (Figure 4), that has been described by some authors as the most active component of ginger, presented a weaker activity in this experiment.[47] The most effective inhibitor **9** also inhibited the interaction of MD-2 with its fluorescent ligand bis-ANS and suppressed both MyD88-dependent NF-κB and AP1 activation (for the expression of TNF-α and IL-1β) and TRIF-dependent pathway for IRF3 activation and expression of IFN-β and IP-10 in LPS-

stimulated macrophages.[47] Despite the low activity for interfering with LPS-MD-2 interaction reported in this paper, the anti-inflammatory activity of 6-shogaol is well established. It has been reported recently that 6-shogaol inhibits LPS-triggered TLR4 homodimerization,[42] and targets TBK1 kinase in the TRIF-dependent pathway.[49]

Roots and rhizomes of *Glycyrrhiza* plants (licorice) have been used as herbal medicine worldwide for over 4000 years. Among *Glycyrrhiza* plants, *G. uralensis* is one of the mostly used in traditional medicines in Japan, and various components have been isolated from *G. uralensis*, including triterpene saponins, flavonoids, isoflavonoids, and chalcones. Glycyrrhizin **12** (GL, Figure 4), a triterpene saponin, and chalcone isoliquiritigenin **13** (ILG, Figure 4), are the major biological active components of licorice. **12** and **13** were reported to suppress TLR4.MD-2-mediated NF-κB and MAPK activation, resulting in decreased production of proinflammatory cytokines.[50] Also, **12** treatment down-regulated the TLR4 internalization upon LPS stimulation.[51] In a recent paper, it was reported that **12** and **13** modulate the initial steps of TLR4 signaling with different mechanisms.[52] While both compounds were active in inhibiting LPS-induced TLR4.MD-2 homodimerization, only **12** and not **13** inhibited the formation of the LPS.MD-2.TLR4 complex in an experiment of coprecipitation with biotinylated LPS.[52] The activity of **13** in inhibiting TLR4 dimerization in a dose-dependent manner was described in another report where the authors propose the formation of a covalent adduct between **13** and MD-2 and/or TLR4 as mechanism of action of ILG.[41] Caffeic acid phenethyl ester **14** (CAPE,[3] Figure 4), first isolated as an active phenolic constituent of honeybee propolis potently inhibits activation of the NF-κB signaling. It has been recently reported that **14** significantly inhibits TNF-induced IP-10 expression in intestinal epithelial cells[55] and that the NF-κB inhibition is due to the formation of a covalent adduct with Cys133 in MD-2 hydrophobic pocket.[43]

Green tea polyphenolic fraction possess anti-inflammatory and chemopreventive effects, and contains several active compounds which are catechin, (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), and **15** (−)-epigallocatechin-3-gallate (EGCG). Among these **15** (Figure 4) is known to possess the most potent anti-oxidative and chemopreventive properties, and it has been demonstrated not to prevent LPS-induced dimerization of TLR4, but to inhibit the TLR4 signal downstream, blocking both MyD88- and TRIF-dependent signal pathways by targeting IKKβ and TBK1 kinases respectively. The parent catechin molecule, showed no LPS induced inhibition of NF-κB activation, thus suggesting an anti-inflammatory property of the galloyl group.[55]

Paclitaxel **16** (PTX,[56] Figure 4) is a potent anti-cancer agent derived from the Pacific yew tree, which acts through over-stabilization of cellular microtubules. Although the anti-cancer mechanism of action of **16** has long been attributed to effects at the level of β-tubulin, growing evidence supports innate immune activation and possibly antitumor effects of **16** mediated through TLR4-MD-2. Activation of the TLR4.MD-2 pathway by **16** has been demonstrated using murine macrophages, transfected cell lines, mouse cancer cell and direct evidence of **16** binding to MD-2 has been collected by *in vitro* experiments.[56] In contrast, cells transfected with human TLR4 and human MD-2 fail to mount a pro-inflammatory response to **16**. On the contrary, recent data show that **16** can block NF-κB activation and cytokines expression by binding to human MD-2 as an antagonist, thus ameliorating TLR4-

dependent pathologies such as LPS-induced kidney injury.[57] The species-specific action of **16** has been explained in terms of different binding to MD-2.[58] **16** binding to murine MD-2 causes a shift of 123–130 loop , including Phe126, outward the protein surface thus creating a binding interface for TLR4 dimerization and formation of the activated TLR4.MD-2 dimer. This repositioning of the loop does not occur when PTX binds to human MD-2, leading to antagonism.[58]

It is known that opioids such as morphine (Figure 4) induce neuroinflammatory responses in CNS, Hutchinson and Watkins recently associated the neuroinflammatory action of morphine to activation of TLR4 signaling. In an elegant work, the molecular mechanism underlying the TLR4 agonist action of morphine **17** has been investigated.[59] Biotinylated morphine immobilized on plate bound to human MD-2 in a dose-dependent manner and LPS competed with immobilized morphine for binding to MD-2. **17** induced TLR4 oligomerization in Ba-F3 cells and proinflammatory cytokines production in TLR4-expressing CNS immunocompetent cells. Interestingly, the co-administration of TLR4.MD-2 antagonists inhibited morphine-induced proinflammatory effect *in vitro* and *in vivo*. The same authors associated the TLR4 agonist action of **17** and opioids to reinforcing/rewarding effects that contribute to the widespread abuse of these drugs.[60, 61] Molecular modeling and docking calculations suggested that, similarly to LPS and **16**, **17** binding to human MD-2 would induce conformational rearrangement of the Phe126 loop, resulting in the formation of the TLR4 oligomerization interface.[59]

The same research group showed that the TLR4 antagonist action of both enantiomers of opioids naloxone (Figure 4) and naltrexone can be used to efficiently contrast acute[62] and chronic[63] neuropathic pain in animal models associated with microglial TLR4 activation.

Chitohexaose **18** (chtx, Figure 4), a small molecular weight oilgosaccharide which is a constituent of some nematode surface glycoproteins, inhibited LPS-induced production of inflammatory mediators by murine macrophages and human monocytes.[64] The authors started from the observation that soluble TLR4 present in membrane lysates of human and murine macrophages binds to solid-phase immobilized parasites of *Setaria digitata* (FAg). This suggested the presence of conserved TLR4 binding components on the surface of nematodes. The hexasaccharide **18**, being a main constituent of helminths surface glycans, was therefore selected as a potential, high-affinity, low-molecular weight TLR4 ligand. Co-administration of **18** blocked LPS-induced lethal endotoxemia in mice, and chtx reversed inflammation and protected mice even 24/48 hrs after onset of endotoxemia. The authors suggest that **18** acts on TLR4 signaling, competing with LPS as an antagonist. Interestingly, it seems that this compound interacts directly with TLR4 without need of MD-2.[64]

A growing number of publications during recent years strongly suggest that the biological activity of some pure natural compounds or extracts from natural sources is related to a specific interaction with MD-2 and/or TLR4.MD-2. The property of the anticancer macrocylic lactone bryostatin (bryo-1) to induce cytokine production in dendritic cells has been recently related to TLR4 agonism,[65] while TLR4 antagonism can explain the inhibitory effect on nitric oxide synthase (iNOS) expression of polysaccharides extracted from pu-erh tea.[66] The anti-inflammatory effect of phenolic compounds such as hydroxytyrosol and

oleuropein aglycon extracted from olives and olive oil is also very likely related with TLR4 antagonism.[67] The identification of active principles in natural extracts and the clarification of the molecular details of the interaction between these molecules and TLR4.MD-2 will provide a variety of chemical scaffolds for developing new, nontoxic, TLR4 modulators.

Conclusions and future directions

The wealth of information available on the structure-activity relationship of natural and synthetic lipid A analogues and the availability of the structures of the human[11] and murine[12] (LPS.MD-2.TLR4)$_2$ complexes, enable the rational design of TLR4 agonists and antagonists based on rational modification of the lipid A structure. This procedure based on LPS (lipid A) mimicry afforded efficient and non-toxic TLR4 agonists MPLAs[18] and AGPs[17] in use as vaccine adjuvants. However rationally designed TLR4 antagonist Eritoran failed in phase III clinical trials.[68] Compound **8** (TAK-242), a small molecule with a chemical structure different from lipid A, and directly targeting TLR4 with a non-classical mechanism of action, also failed to suppress cytokines levels in patients in a phase III clinical trial. The lack of activity in septic patients of both these TLR4 antagonists, that were very efficient in preclinical phase on animal models, may imply that the current approaches to target the TLR4 receptor should be part of a more general approach to the sepsis. Expression of TLR4 varies in different stages of sepsis, in particular TLR4 expression on neutrophils in septic shock patients is down-regulated in the acute phase.[69] Very recently, a TLR4-independent mechanism for innate immune recognition of LPS and LPS-induced inflammation has been observed.[70] These data could explain the lack of efficacy of TLR4 inhibition in septic patients. Novel, integrated, multi-targeted pharmacological strategies are therefore urgently needed to regulate the innate immune response.

From a chemical point of view, the amphiphilic character of all synthetic and natural lipid A analogues is often associated with low solubility in aqueous media. In this respect, the natural TLR4-active compounds reviewed in this article have better water solubility and bioavailability. The chemical modification of compounds such as caffeic acid esters, isoliquiritigenin, shogaol, gingerdione, curcumin, opioids and taxanes should therefore afford new generations of TLR4-active compounds with improved pharmacokinetic properties and reduced toxicity. Synthetic modification of natural compounds is required 1) to increase target-specificity, and 2) to improve water stability and resistance to enzyme hydrolysis.

Detailed molecular modeling and docking studies are available on the interaction of MD-2 with agonists **16**[58] and **17**.[59] These models suggest a common mechanism based on the displacement of Cys126 loop to trigger TLR4 dimerization and activation. The ligand-MD-2 complex structures from these studies should guide the design of new MD-2 ligands with agonist or antagonist activity based on natural scaffolds.

Some natural compounds presented in this review act as disruptors of the TLR4.MD-2 interaction by forming covalent bonds with Cys133 of MD-2. An appealing and still unexplored way to have access to novel, high-affinity TLR4 antagonists, would be the incorporation of thiol (cysteine)-reactive electrophile groups into synthetic or natural TLR4

ligands. This would combine the high target specificity with increased affinity due to covalent bond formation. Finally, no natural ligands have been so far co-crystallized with the MD-2.TLR4 complex. The use of NMR for the determination of ligand.TLR4.MD-2 complexes structure has so far been limited by the availability of pure and functional TLR4 and MD-2 receptors and by the poor aqueous solubility of both receptors and ligands at the high concentrations required for NMR experiments. Once addressed the practical issues cited above, NMR experiments such as Saturation Transfer Difference (STD) and transfer-NOE would provide atomic-level structural information thus allowing to map the regions of the synthetic or natural ligand directly interacting with MD-2.TLR4 (epitope mapping) and *vice-versa* the residues of MD-2.(TLR4) involved in ligand binding and recognition. Some recent reports on the determination of the structures of[13]C-enriched lipooligosacharide (LOS) with wild-type MD-2 or MD-2 mutants[13] suggest the possibility to prepare[13]C-enriched synthetic or natural molecules for similar binding studies.

A multidisciplinary effort is needed to gain information on the atomic structure of different ligand-MD-2.TLR4 complexes, thus enabling the rational design of non-classical TLR4 modulators.

Acknowledgments

Abbreviations

AGP	aminoalkyl glucosaminide 4-phosphates
	Ba/F3 cells
CD14	cluster differentiation antigen (14)
CNS	central nervous system
DAMP	Danger Associated Molecular Pattern
E	endotoxin
FA	fatty acid
	HEK cells
HMGB1	high-mobility group protein 1
HSP	heat shock protein
LBP	lipopolysaccharide binding protein
LOS	lipooligosaccharide
LPS	lipopolysaccharide
MD-2	myeloid differentiation protein (2)

MPL	monophosphoryl lipid A
MyD88	myeloid differentiation primary response gene (88)
NF-κ	nuclear factor kappa-light-chain-enhancer of activated B cells
TNF-α	tumor necrosis factor alpha
PAMP	pathogen associated molecular pattern.

Biographies

Francesco Peri

Francesco Peri was born in San Francisco (USA) in 1968. He received a first PhD in Chemistry at the University of Parma (Italy) in 1998 and a second PhD in Bioorganic Chemistry at the University of Lausanne (Switzerland) in 2001. Since 2007 he is Professor of Organic Chemistry at the Department of Biotechnology and Biosciences, University of Milano-Bicocca (Italy). He is visiting professor at the Ecole Normale Superieure of Lyon (France) and at the Department of Chemistry, University of Davis (CA). His research interests span the disciplines of organic, bioorganic and medicinal chemistry with an emphasis of study of the interactions between small molecules and protein targets. At present, his research is focused on the study of molecular mechanisms of innate immunity and inflammation.

Valentina Calabrese

Valentina Calabrese graduated in Chemistry in 2005 at the University of Milan, in 2008 she received the PhD degree in Industrial Chemistry. In 2009 she was appointed as post-doc in the research group of F. Peri at the University of Milano-Bicocca. Her research focuses on the synthesis and biological characterization of synthetic TLR4-active compounds with the aim to develop new anti-sepsis drugs (TLR4 antagonists) and vaccines adjuvants (TLR4 agonists). Several active compounds have been developed with good activity in TLR4 modulation through selective CD14 and MD-2 co-receptors targeting, and low in vitro toxicity. Some of these new compounds are in preclinical phase as anti-inflammatory and anti-neuropathic pain drugs.

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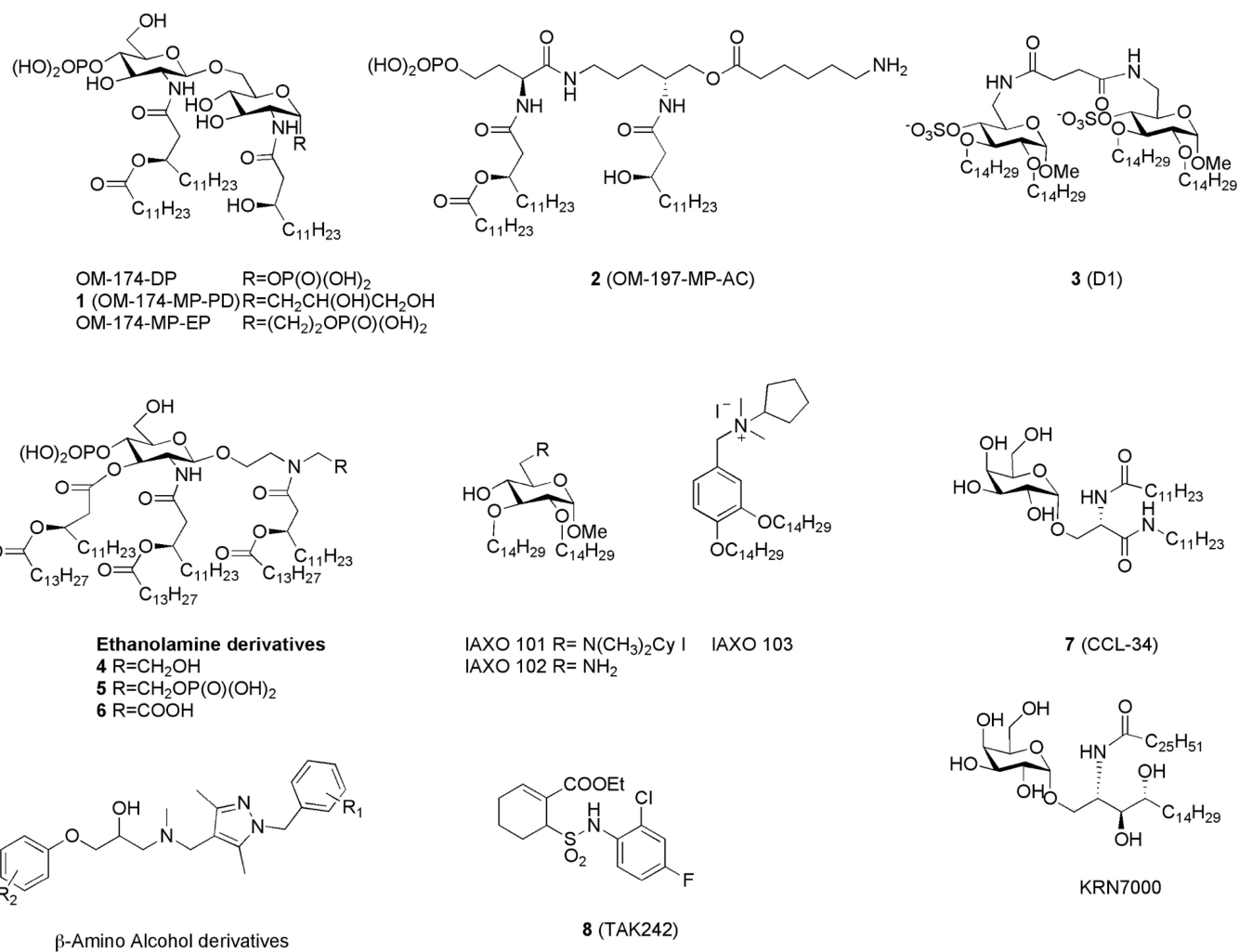
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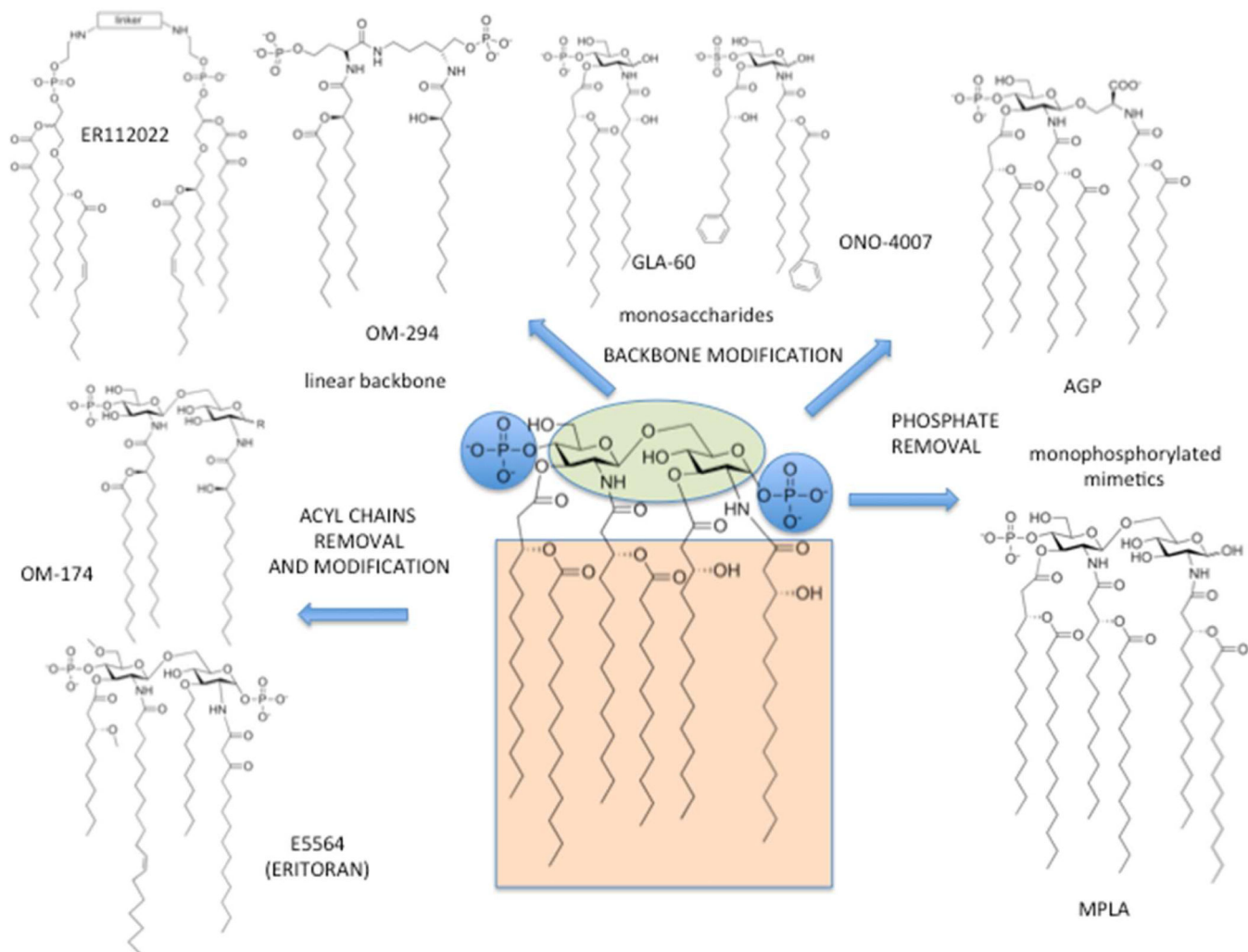
Figure 1.
TLR4 agonists and antagonists obtained by chemical elaboration of *E. coli* lipid A structure.
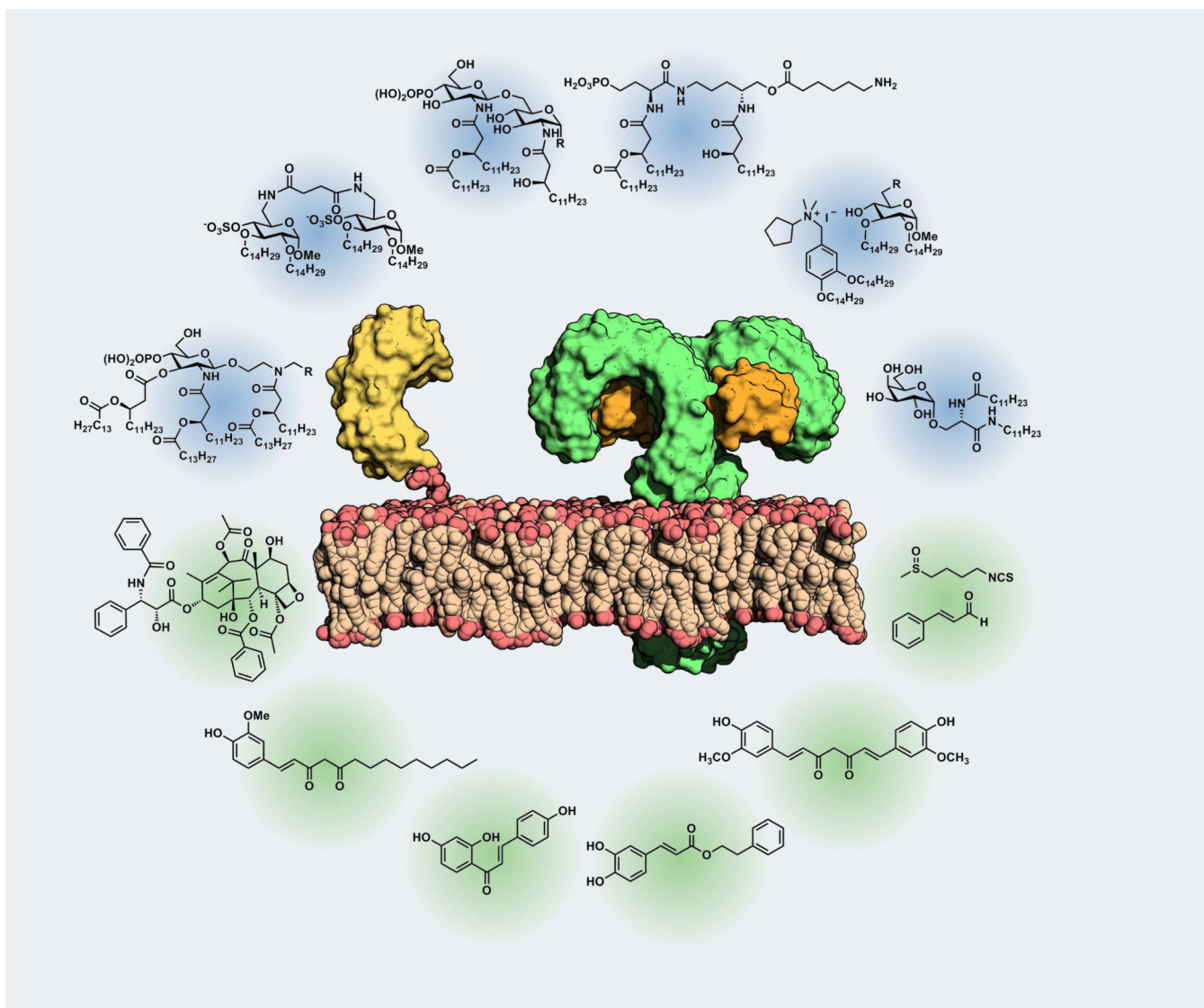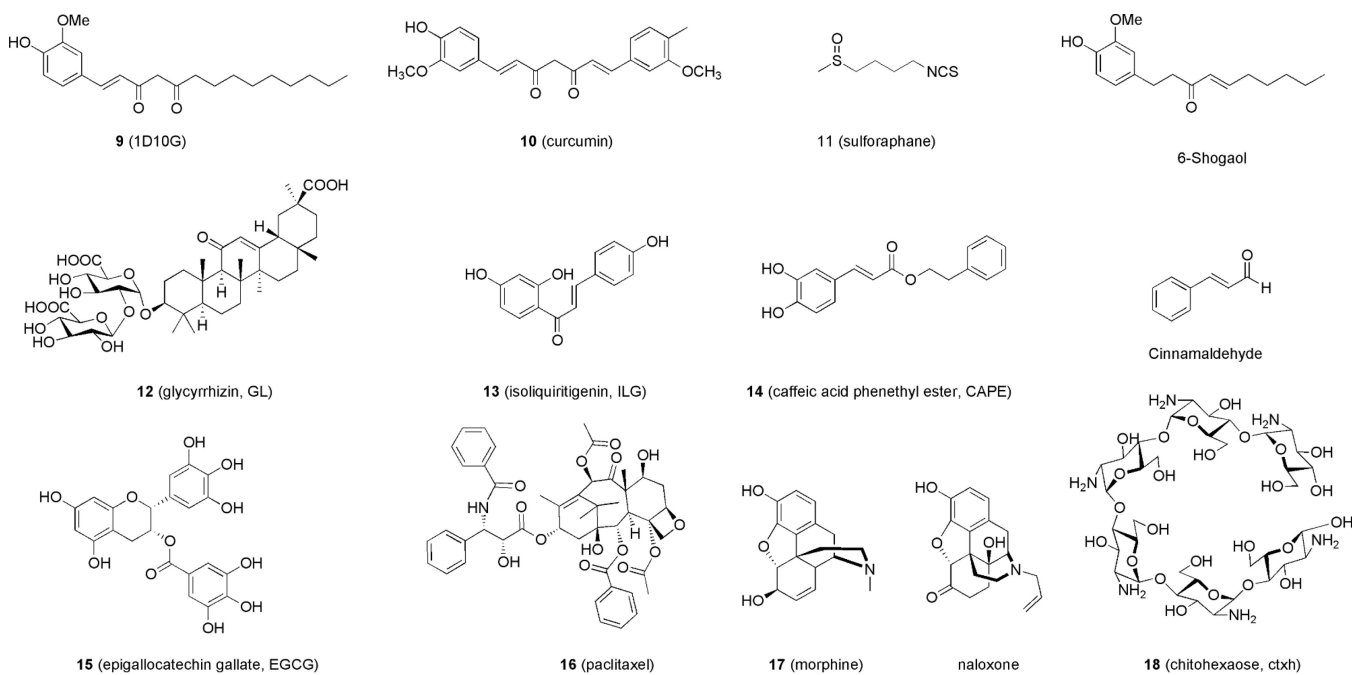
Figure 2.
Pictorial representation of the TLR4 receptor complex (including MD2 and CD14 co-receptors) and some natural (green background) and synthetic (blue background) TLR4 ligands.

Figure 3.
Recently developed synthetic TLR4 modulators (agonists and antagonists).

9 (1D10G)

10 (curcumin)

11 (sulforaphane)

6-Shogaol

12 (glycyrrhizin, GL)

13 (isoliquiritigenin, ILG)

14 (caffeic acid phenethyl ester, CAPE)

Cinnamaldehyde

15 (epigallocatechin gallate, EGCG)

16 (paclitaxel)

17 (morphine)

naloxone

18 (chitohexaose, ctxh)

Figure 4.
Some recently studied TLR4-active compounds from natural sources.