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Original article

Novel toll-like receptor 4 (TLR4) antagonists identified by structure- and ligand-based virtual screening

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ABSTRACT

Toll-like receptor 4 (TLR4) in complex with its accessory protein MD-2 represents an emerging target for the treatment of severe sepsis and neuropathic pain. We performed structure-based and ligand-based virtual screening targeting the TLR4–MD-2 interface. Three *in silico* hit compounds showed promising TLR4 antagonistic activities with micromolar IC_{50} values. These compounds also suppressed cytokine secretion by human peripheral blood mononuclear cells. The specific affinity of the most potent hit was confirmed by surface plasmon resonance direct-binding experiments. The results of our study represent a very promising starting point for the development of potent small-molecule antagonists of TLR4.

1. Introduction

The first line of immunological defense against microbial pathogens is mediated through innate immune signaling via various pattern-recognition receptors (PRRs). Although lacking antigen specificity, the relative simplicity of the innate immune system enables the induction of a rapid and extensive immune response through recognition of pathogen-associated molecular patterns (PAMPs). In this context, the toll-like receptors (TLRs) are among the most important PRRs, as they recognize a wide variety of PAMPs from bacteria, viruses, fungi, and parasites [1].

Among the more than 10 human TLRs already identified, TLR4 is one of the most studied and clinically relevant of the PRRs, due to its involvement in several immune-mediated pathologies. It is located on the cell surface and recognizes microbial components, such as lipopolysaccharide (LPS) [2]. This recognition of LPS is mediated through the heterodimerization of TLR4 with its accessory protein myeloid differentiation factor 2 (MD-2), which initiates an intracellular signaling cascade that can activate both Myd88-dependent and TRIF-dependent pathways [3]. This leads to activation of the Nf-κB and IRF3 transcription factors, and the subsequent expression of inflammatory cytokines and activation of innate immune cells, such as macrophages and dendritic cells.

Although LPS-induced proinflammatory cytokine production initiates the host defense system against injury and infection, the dysregulation of TLR4 signaling has been directly implicated in an array of acute and chronic human diseases, such as sepsis and neuropathic pain [4,5]. The importance of TLR4 in the initiation of immune responses makes it an ideal therapeutic target for potential immune-intervention drugs.

Unsurprisingly, several TLR4 antagonists have already been investigated as potential antisepsis drugs (Fig. 1). The most advanced of these, TAK-242 and eritoran, were successful in pre-

Abbreviations: HEK293, human embryonic kidney 293 cells; LPS, lipopolysaccharide; MD-2, myeloid differentiation factor 2; PAMPs, pathogen-associated molecular patterns; PBMCs, peripheral blood mononuclear cells; PPI, protein–protein interaction; PPRs, pattern-recognition receptor; SEAP, secreted embryonic alkaline phosphatase; SPR, surface plasmon resonance; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor-α.

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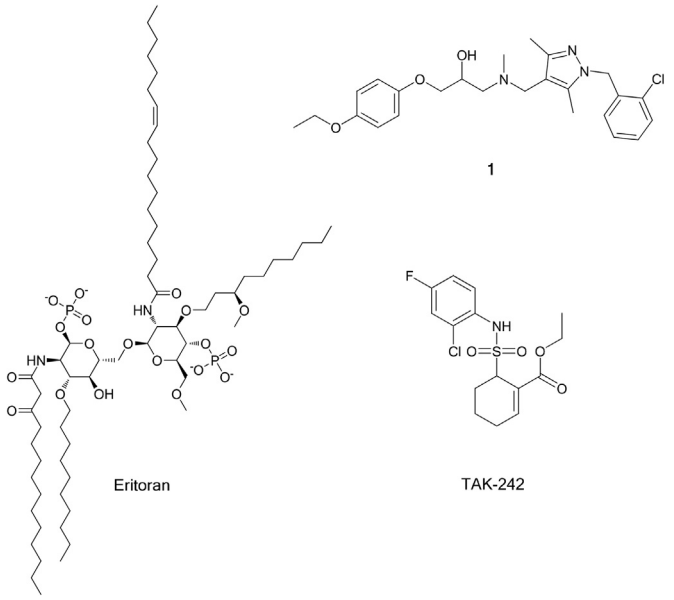


Fig. 1. The known TLR4 antagonists.

clinical trials. Unfortunately, both strategies failed to meet its primary endpoint to reduce the mortality rate in patients with sepsis [6,7]. TAK-242 is a cyclohexene derivative that inhibits the production of LPS-induced inflammatory mediators by binding to the intracellular domain of TLR4 [8], while eritoran mimics Lipid-A and binds to a large hydrophobic internal pocket of MD-2 [9].

Small-molecule modulation of protein–protein interactions (PPIs) is one of the most exciting yet challenging fields in chemical biology and drug development. The most recent estimation about the total number of PPIs in an organism is centered to about 300,000 [10]. The PPIs targets have traditionally been shunned by many small-molecule drug developers, despite their therapeutic relevance and untapped abundance. This was mainly due to the belief that the interfaces between two proteins were too large and featureless for small molecules to be able to act effectively. But this view has evolved and several small-molecule PPI inhibitors have already been discovered [11,12].

The recently reported crystal structures of the TLR4–MD-2 complex have revealed that the surface of TLR4 that interacts with MD-2 has a long and narrow shape, with dimensions of 40 Å × 20 Å. This surface can be divided into two chemically and evolutionarily distinct areas: the A and B patches [9,13]. The protein–protein interactions between TLR4 and MD-2 are mediated by an extensive network of charge-enhanced polar contacts. The most important of these are the hydrogen bonds between Ser-183 and Asp-209 of TLR4 and Arg-106 of MD-2 [14]. The solved crystal structures and the knowledge of the crucial protein–protein interactions have enabled the structure-based discovery of new antagonist of TLR4. The β-amino alcohol derivatives have recently been identified as inhibitors of the TLR4-mediated inflammatory response, apparently by disrupting the formation of the TLR4–MD-2 complex [15]. However, the most active compounds (e.g., compound **1**, Fig. 1) showed significant cytotoxicity towards HEK293 cells (see Supporting information of [15]). Therefore, the identification of new TLR4 antagonists that can serve as novel therapeutics still remains an urgent need.

In the present study, we report on the successful application of ligand-based and structure-based virtual screening in the discovery of novel TLR4 antagonists that target the TLR4–MD-2 interface. In this work, three hits were identified to show promising antagonistic activity in the reporter assay. Furthermore, all three hits suppressed cytokine secretion by human peripheral blood mononuclear cells. The specific affinity of the most potent hit compound **3** was confirmed by surface plasmon resonance direct-binding experiments. This compound thus represents a very promising starting point for the development of potent small-molecule antagonists of TLR4, with potential for treatment of important diseases such as sepsis and neuropathic pain.

2. Materials and methods

2.1. Virtual screening of the compound library and molecular docking into the TLR4–MD-2 interface

2.1.1. Computer hardware and compound database preparation

The computational study was carried out on a workstation with four dual core AMD Opteron 2.0 GHz processors, 16 GB RAM, four 320 GB hard drives in a RAID10 array, and Nvidia GeForce 7900 graphic cards, running 64-bit Fedora 7. For both, ligand-based and structure-based virtual screening, the ZINC drug-like subset was used, which now includes 11.3 million drug-like compounds [16]. To cover as much conformational space as possible, the database was first processed with the Omega 2.4.3 software (OpenEye Scientific Software, Santa Fe, NM.) [17,18] using default settings, which yielded an average of 152 conformations per compound.

2.1.2. Ligand-based virtual screening

For the ligand-based three-dimensional (3D) similarity search compound **1**, a known TLR4 antagonist, was used as a query molecule [15]. Omega 2.4.3 software was used to generate 5 conformers of compound **1**. ROCS 3.1.1 (OpenEye Scientific Software, Santa Fe, NM) [19] using default settings was used for comparing molecules in ZINC drug-like database to all query conformers. The single best overlay hits were ranked according to the Tanimoto-Combo scoring function, which considers similarities in the molecular shape and color (atom types). From the compounds with the highest similarity to compound **1**, 5 available compounds were evaluated *in vitro* (see Table S1, compounds S41–S45, Supporting information).

2.1.3. Structure-based virtual screening

To reduce and enrich the ZINC drug-like subset for the structure-based virtual screening (SBVS), 3D shape comparisons between compound **1** and the molecules in the ZINC drug-like subset were first performed using ROCS. Here, 25,000 compounds with the highest shape similarities (ShapeTanimoto) and 25,000 compounds with the highest shape and color similarities (TanimotoCombo) to compound **1** were retained and merged into the single focused compound library. Python script (see Supporting information for the Python script) was used to check for the overlap of both libraries in order to remove duplicates. Docking was carried out using the LeadIT 2.1.2 software (BiosolveIT GmbH) [19] and the crystal structure of the human TLR4–MD-2 complex with the bound endotoxin antagonist Eritoran (PDB code: 2Z65) [8]. The active site was defined as an area of TLR4 within 8 Å around the crucial interacting MD-2 loop (Gly-97–Leu-108). The docking experiment was set up with LeadIT-implemented pharmacophore constraints, which were selected to keep only the compounds that can form interactions with at least one of the polar amino-acid residues (Asp-209, Ser-183). All of the 49,600 unique compounds from the ROCS enriching procedure were docked into the active site of TLR4 and ranked according to their best scoring conformation. Forty of those compounds were available and subsequently evaluated in *in vitro* experiments (see Table S1, compounds S1–S40, Supporting information).

2.2. Compound chemical characterization

We assessed the purity of active hit compounds **3**, **6** and **33** using reversed-phase high-performance liquid chromatography (HPLC) analysis on an Agilent 1100 system (Agilent Technologies, Santa Clara, CA, USA) equipped with a quaternary pump and a multiple-wavelength detector, using an Agilent Eclipse Plus C18, 5 μm column (4.6 × 50 mm, 5 μm), with a flow rate of 1.0 mL/min, detection at 254 nm, and an eluent system of: A = 0.1% TFA in H_2O; B = MeOH. The following gradient was applied: 0–20 min, 10% B → 90% B in A; 20–25 min, 90% B in A; 25–30 min, 90% B → 10% B in A. The runtime was 30 min, at a temperature of 25 °C. The relative purity of all of the active compounds was above 95.0% as determined by HPLC.

^{1}H-NMR spectra were recorded on a Bruker AVANCE 400 DPX spectrometer (400 MHz for 1H) at 298 K. Chemical shifts are reported in ppm downfield from tetramethylsilane (δ 0.00 ppm) or solvent (DMSO-d6 at 2.50 ppm, CDCl3 at 7.26 ppm) as an internal standard. The coupling constants (J) are in Hz, and the splitting patterns are designated as: s, singlet; br s, broad singlet; d, doublet; dd, double doublet; ddd, triple doublet; t, triplet; dt, double triplet; and m, multiplet. Mass spectra were obtained using a VG-Analytical Autospec Q mass spectrometer. Results of compound characterization are available in S2, Supporting information.

2.3. In vitro and cell-based assay

2.3.1. Solubility and cytotoxicity studies

25 mM stocks of the compounds were prepared in DMSO and tested for their solubility in cell-culture medium (RPMI 1640). Solubility was confirmed by using an inverted light microscope (Nikon Eclipse TE300, Tokyo, Japan), to examine the solutions for precipitation. Cytotoxicity of the soluble compounds was assessed using the same cell line that was used later for the TLR4-reporter assay, HEK293 cells (Invivogen, San Diego, CA, USA). Briefly, the cells were seeded in the wells of 96-well plates and cultured in the absence and presence of the compounds at 100 μM or 200 μM. Pure DMSO was used for the controls. After 24 h culture, the cells were harvested and stained with propidium iodide (Invitrogen, Carlsbad, CA, USA) to monitor for dead cells. The results were analyzed by flow cytometry.

2.3.2. TLR4 reporter assay

To specifically evaluate the inhibitory activity of the selected compounds towards TLR4 signaling, we used a HEK-BlueTM-hTLR4 reporter cell line (Invivogen), which selectively expresses the TLR4 receptor and activates secreted embryonic alkaline phosphatase (SEAP) as a reporter gene, via TLR4 agonists. The antagonism of these compounds was assessed as indicated by the manufacturer. The negative control comprised only the TLR4 agonist lipopolysaccharide (LPS) (Sigma–Aldrich, St Louis, MO, USA). For the positive control, we used the commercially available TLR4 inhibitor TAK-242 (Invivogen), according to the manufacturer instructions. To evaluate the antagonistic effects of our compounds, the cells were cultured as specified by the provider. The cells were then pre-incubated with these compounds for 2 h at 37 C at various concentrations. Afterward, the cells were challenged with LPS for 20 h. The next day, the supernatants were sampled and analyzed colorimetrically for the presence of SEAP. The IC_{50} values of the compounds were calculated based on their inhibition of SEAP expression in the media; e.g., by a decrease in the absorbance measurement.

2.3.3. Cytokine secretion assay

The active compounds from reporter assay were further used in a cytokine secretion assay with human peripheral blood mononuclear cells (PBMCs). Briefly, PBMCs were isolated from the blood of healthy volunteers at the Blood Transfusion Centre of Slovenia. The cells were then pre-incubated with compounds **3**, **6** and **33** at 100 μM for 2 h, and afterward they were challenged with LPS for 48 h. TAK-242 was used as the positive control. After 2 days, the supernatants were sampled and analyzed for the presence of tumor necrotic factor (TNF)-α using an ELISA.

2.4. Surface plasmon resonance spectroscopy

Surface plasmon resonance (SPR) experiments were performed using a Biacore T100 (GE Healthcare) equipped with a Series S sensor chip CM5 (GE Healthcare). HBS-EP buffer (10 mM Hepes, 150 mM NaCl, 0.005% P20, pH 7.4) was used for immobilization of the protein. Recombinant human TLR4 (R&D Systems) was attached to the surface of the chip by amine coupling. The surface of flow cells 1 and 2 was initially activated with a 12-min pulse of a 1:1 mixture of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride and 0.1 M N-hydroxysuccinimide, according to manufacturer recommendations. TLR4 was diluted into 10 mM sodium acetate buffer, pH 5.0, to a final concentration of 200 μg/mL, and injected twice for 600 s over the second flow cell. Both flow cells were blocked with a 7-min pulse of 1.0 M ethanolamine. The final immobilization level was about 8000 RU. Screening was run at 25 °C using PBS (10 mM Na2HPO4, 2 mM KH2PO4, 137 mM NaCl, 2 mM KCl, pH 7.4) supplemented with 2% DMSO (Merck) as the running buffer. All of the three hits from the cell-based assays were tested at two different concentrations: 20 μM and 200 μM. Each compound was injected for 1 min at a flow-rate of 30 μL/min, and the dissociation was monitored for 30 s. Regeneration was not needed, although 30 s of buffer-flow was used to stabilize the surface after each injection. We compared the responses obtained by the injections of the buffer and the two different sample concentrations, and continued the study with the compound that showed binding. We used the following concentrations for titration: 0, 5, 10, 20, 40, 80 (repeated at the end of the concentration series), 160, and 320 μM.

3. Results and discussion

3.1. Virtual screening

We used two parallel VS approaches in the search of novel TLR4 antagonists. As the β-amino alcohol derivatives with TLR4 antagonistic activity were discovered by a virtual screening of 700.000 compounds in ENAMINE screening collection (see Supplementary materials of [20]), this time a larger ZINC drug-like subset was used to further explore a chemical space around compound **1**. Five compounds with the highest similarity to compound **1** according to ROCS software were purchased (see Table S1, compounds S41–S45, Supporting information, for their structures and scores) and evaluated *in vitro*.

Previous *in silico* simulations predicted, that the β-amino alcohol derivatives recognize the same cleft on the surface of TLR4 to which a protruding loop region of MD-2 binds. These results suggest that β-amino alcohol derivatives may inhibit the TLR4-signaling pathway by disrupting the TLR4–MD-2 complex formation by competing with MD-2 binding to TLR4 [20,21]. Given the importance of the shape of a molecule in molecular recognition, a rational strategy would be to incorporate the knowledge about the known ligands into the design of the focused compound library [22]; therefore, as the first step in the structure-based virtual screening, the ZINC drug-like subset of 11.3 million compounds was enriched using ROCS and compound **1** as a query for 3D similarity search. Two parallel 3D similarity searches were performed with one considering only shape and the other considering both shape

and atom types (dubbed color). From each 3D similarity search, 25.000 compounds were retained and checked for the overlap between both new libraries. Only 400 compounds were shared and merging of both libraries thus yielded a focused library of 49.600 unique compounds. This focused library was further docked in the TLR4–MD-2 interface. The preparation of the active site was carefully planned and the nature of protein–protein interactions between TLR4 and MD-2 was considered. The active site was defined as the area of TLR4 within 8 Å from the MD-2 Gly-97–Leu-108 loop. This loop forms crucial interactions with TLR4, especially with polar Ser-183 and Asp-209. To increase the chance of hits successfully mimicking interactions with MD-2, pharmacophore constraints with at least one of the mentioned residues were set up. The 25.750 compounds satisfied the implemented pharmacophore constraints and were successfully docked into the defined active site. Compounds were ranked according to the LeadIT score. Available forty top-ranked compounds were purchased and evaluated *in vitro* (see Table S1, compounds S1–S40, Supporting information, for their structures and scores). As expected, docking yielded structurally more diverse compounds than the ligand-based 3D similarity search. The complete enriching and screening procedure is depicted in Scheme 1.

Among the 45 selected compounds, only 18 were sufficiently water-soluble, up to 500 μM. The poor solubility of the screening hits was a major problem, and consequently a valuable lesson was learned, such that algorithms for predicting aqueous solubility will be integrated into future virtual-screening campaigns. Among the 18 water-soluble compounds, 14 were completely non-cytotoxic at 100 μM, compared to the DMSO control. These compounds were then further used for biological evaluation.

3.2. Measurement of in vitro TLR4 antagonistic activity

When assessing the biological activity of receptor ligands, the most common and assuring approach is to first use a reporter assay cell line, before proceeding to evaluation on primary target cells of interest. For this purpose we used commercially available HEK-BlueTM-hTLR4 cells, which are obtained by co-transfection of the human TLR4, MD-2 and CD14 co-receptor genes, and an inducible SEAP (secreted embryonic alkaline phosphatase) reporter gene into HEK293 (human embryonic kidney) cells. The advantage of using such an approach is the selective expression of TLR4 by HEK293 cells, thereby greatly excluding false positive results with other types of PRRs.

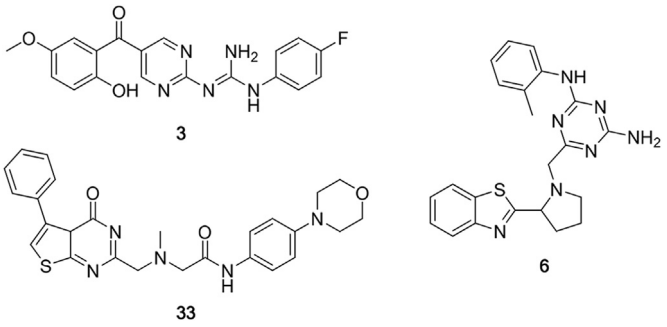
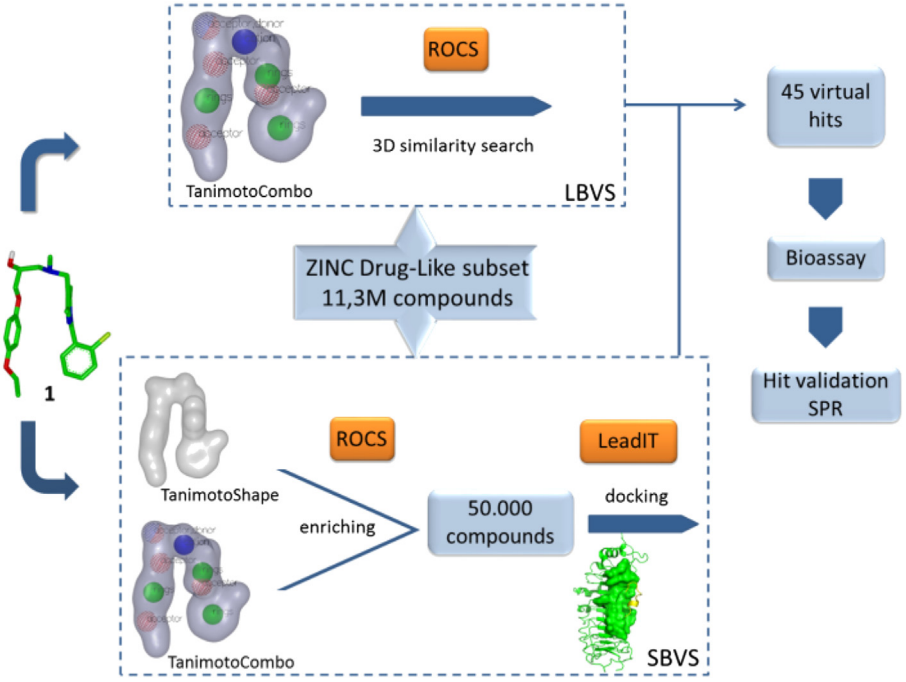


Fig. 2. Structures of the hit compounds identified by the virtual screening.

Out of the fourteen *in silico* hits tested, compounds **3**, **6** and **33** showed promising antagonistic activities towards TLR4 (Fig. 2). The IC_{50} values for the hits ranged from 16.6 μM to 167.5 μM (Table 1). The most potent of these was compound **3**, with an IC_{50} of 16.6 μM (Fig. 4A). Since the most active β-amino alcohol derivative (compound **1**), recently identified as a potent TLR4–MD2 complex inhibitor, showed significant cytotoxicity against the HEK293 cell line (see Supporting information of [15]), our results are the first to demonstrate a specific biological activity of a small-molecule TLR4 inhibitor by a reporter assay.

LeadIT scored compound **3** with a binding energy of −31.70 kcal/mol, which was one of the best scores overall. According to the docking experiment, compound **3** is predicted to bind to the negatively charged A patch of TLR4 by forming multiple H-bonds between the guanidine moiety of compound **3** and amino-acid residues Asp-234, Asp-209, and Ser-211 of the A patch (Fig. 3).



Scheme 1. Overview of the virtual screening work-flow for the identification of novel TLR4 antagonists.

Table 1
Biological evaluation data for compounds **3**, **6** and **33**.

Compound	IC50 ± SEM (μM)	%*I* of TNF-α secretion at 100 μM	LeadIT score (kcal/mol)
3	16.6±1.2	87.4	−31.70
6	64.1±1.4	80.8	−30.83
33	167.5±1.4	93.3	−25.42

Hit compound **6** showed an IC_{50} of 64.1 μM and was scored slightly lower, although the docking predicted polar contacts between its amino group and negatively charged amino-acid residues of TLR4. Compound **33** was the least potent in the LPS-induced SEAP assay, possibly because it cannot form polar contacts with crucial amino-acid residues, as predicted by LeadIT (see Fig. S1, Supporting Information for docking figures). All of these three hits were identified by the structure-based virtual screening, whereas compounds from the 3D similarity search proved to be either non-soluble or cytotoxic to HEK293 cells.

3.3. Measurement of cytokine secretion inhibition

To further assess TLR4 antagonistic activity, we evaluated the compounds **3**, **6** and **33** in cytokine secretion assay with human peripheral blood mononuclear cells. PBMCs represent an important cell population for biological evaluation of immunomodulatory compounds since they contain all major leukocytes, including those that express TLR-4, particularly the dendritic cells and macrophages. Both these cell types are rapidly activated upon TLR-4 stimulation and produce increased quantities of pro-inflammatory cytokines like TNF-α [23]. Consistent with the results obtained in the reporter assay, compound **3** showed strong activity and reduced TNF-α secretion by the PBMCs, to 12.6% at 100 μM, whereas compound **6** showed lower potency. Surprisingly, compound **33** exhibited one of the strongest antagonistic activities on TNF-α secretion, despite showing a lower activity in the reporter assay. This suggests that compound **33** most likely binds to other biological targets in the inflammatory cascade during PBMC activation (Fig. 4B).

3.4. Hit validation by SPR

Since the docking experiments suggested a direct binding of the hit compounds to the TLR, this possibility was investigated through Biospecific Interaction Analysis using the SPR-based biosensor Biacore T100 (GE Healthcare). Recombinant human TLR4 was immobilized on the chip, and the compounds were tested at two different concentrations: 20 μM and 200 μM. At a 20 μM concentration of the tested compounds an increase in SPR signal (expressed in response units, RU) was observed, indicating binding to the immobilized TLR4; however, according to the SPR data, compounds **6** and **33** were aggregated at higher concentrations due to poor solubility in the SPR running buffer (Fig. 5).

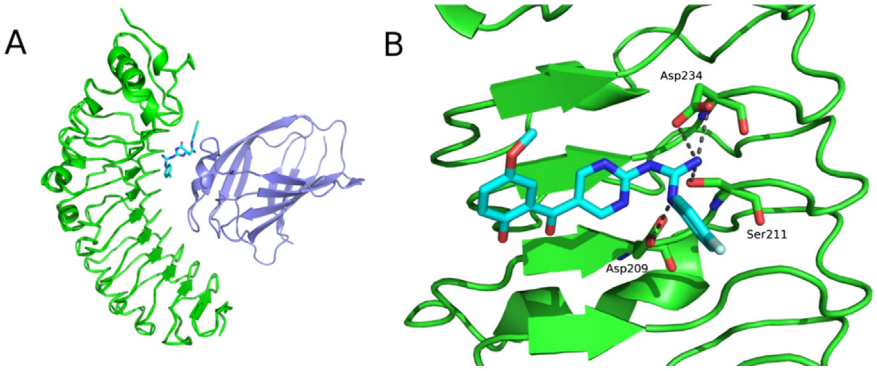


Fig. 3. Predicted binding position of compound **3** (cyan) on TLR4 (green). (A) The interaction with the crucial amino-acid residues may disrupt protein–protein interaction with MD-2(violet). (B) Predicted interactions of **3** with the TLR4. The amino-acid residues important for interactions with ligands are shown as green sticks. The H-bonds with Asp-209, Ser-211, and Asp-234 are shown as gray dashes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

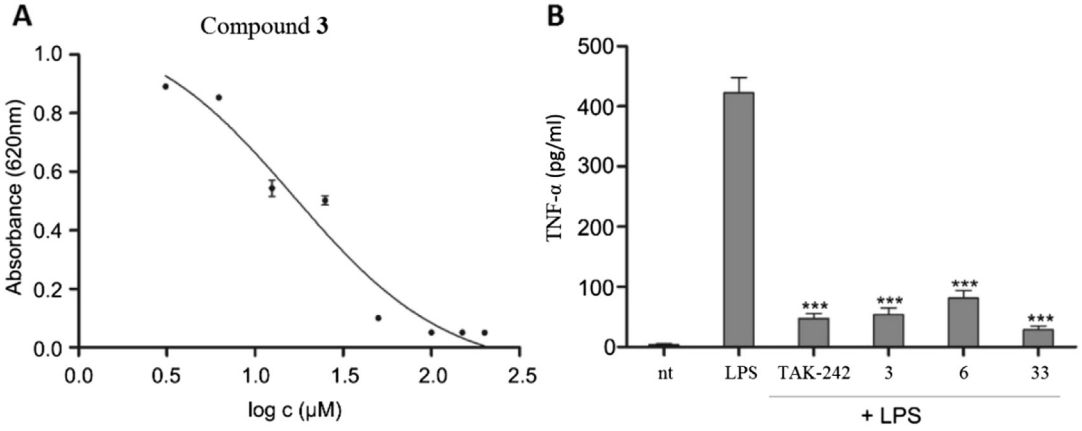


Fig. 4. Biochemical analysis of the hit compounds. (A) Concentration–response curve for inhibition of LPS-induced SEAP activity by hit compound **3**. (B) Inhibition of LPS-induced TNF-α secretion from PBMCs by hit compounds **3**, **6** and **33** at 100 μM. The statistical significance between individual pairs (treated vs. LPS control) was determined using Student's unpaired *t* tests. (***, $p < 0.001$).

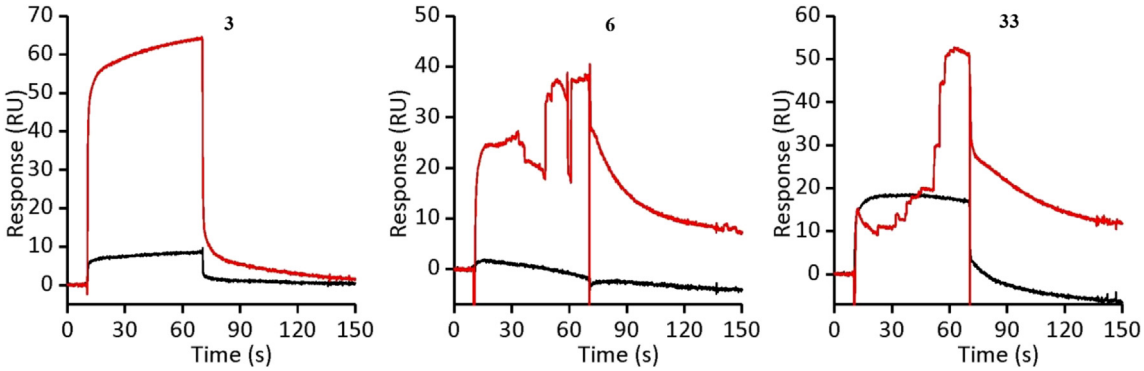


Fig. 5. Binding of the compounds to the chip-immobilized TLR4. Two different concentrations of each compound were probed (20 μM, black; 200 μM, red). Compounds **6** and **33** aggregated at the higher concentration. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

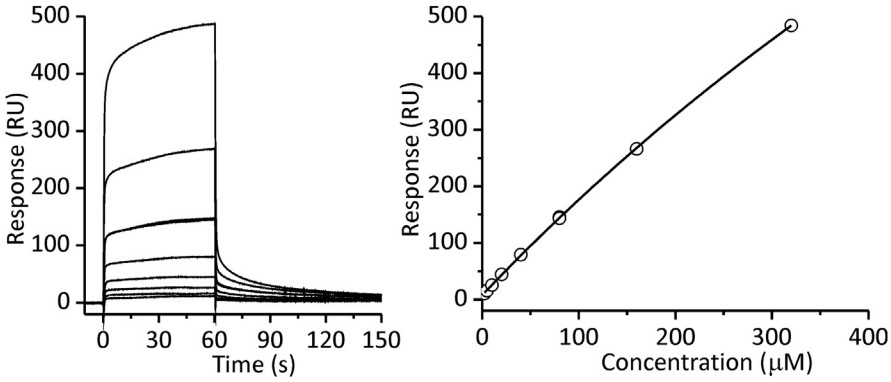


Fig. 6. Binding of compound **3** to TLR4. Compound **3** was injected at concentrations from 5 μM to 320 μM (using two-fold dilutions; left panel, from the bottom to top), with 80 μM used twice for comparisons. The responses at the end of the association phase are reported for each concentration used on the right panel.

Compound **3** was further analyzed using a series of concentrations up to 320 μM (Fig. 6). There was clear binding of compound 3 observed, with rapid association and dissociation rates. However, saturation of TLR4 was not achieved, as the compound **3** is poorly soluble in the running buffer at concentration higher than 320 μM, and therefore it was not possible to estimate the equilibrium binding constant.

4. Conclusions

In summary, we performed a successful structure-based virtual screening of a ZINC drug-like subset targeting the TLR4–MD-2 interface. From 45 virtual hits three novel TLR4 antagonists were identified using a reporter assay. Compound **3** was the most promising hit compound, with an IC_{50} value of 16.6 μM and no cytotoxic properties. All three TLR4 antagonists also significantly and extensively prevented the production of TNF-α from PBMCs upon LPS-induced activation. To confirm the direct binding and affinity of the active compounds to the TLR4, a surface plasmon resonance was used. A clear binding of compound **3** was observed, with rapid association and dissociation rate, thus confirming the biological results with biophysical method. We expect that our strategy of simple hierarchical virtual screening with subsequent biochemical and biophysical assays may be generally applicable in virtual screening campaigns targeting protein–protein interactions. The identified hits represent a very promising starting point for the development of potent small-molecule antagonists of TLR4, with potential for treatment of important diseases such as sepsis and neuropathic pain.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.10.019.

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