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Application of a novel in silico high throughput screen to identify selective inhibitors for protein–protein interactions

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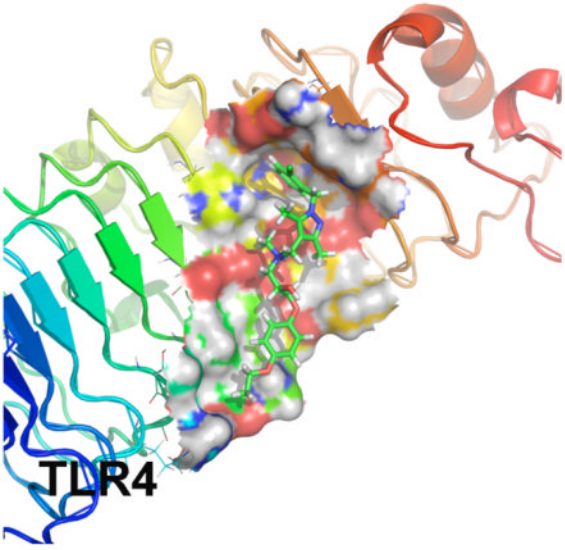
Abstract



Increasing numbers of target protein structures available for computational studies makes the structure-based screening paradigm more attractive for initial hit indentification. We have developed a novel in silico screening methodology incorporating Molecular Mechanics (MM)/ implicit solvent methods to evaluate binding free energies and applied this technology to the identification of inhibitors of the TLR4/MD-2 interaction.

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Keywords

virtual screening; protein-protein interactions; TLR4; MD-2

Lead generation is a pivotal stage in the drug discovery process. Potential lead compounds are generally identified by screening of large compound libraries, i.e. high-throughput screening (HTS). Experimental methods are limited by the cost and complexity of assay preparation and screening quality issues. Moreover, HTS assays are often limited to commercially available compound libraries. Currently, computational screening methods, such as ligand- and structure-based screening, though prone to their own particular drawbacks, can at least complement experimental HTS procedures. Rapidly increasing numbers of target protein structures available for computational studies makes the structure-based screening paradigm more and more attractive for initial hit identification. As time and research progresses, in silico methods may demonstrate even more importance in early stage drug discovery. In this paper, we report progress towards that goal. We have developed a novel in silico screening methodology incorporating Molecular Mechanics (MM)/implicit solvent methods to evaluate binding free energies and applied this technology to the identification of inhibitors of the TLR4/MD-2 interaction. The identified small molecule agents have been evaluated using established whole cell assays to demonstrate potency and selectivity.

We decided to challenge our methodology to identify small molecule inhibitors for the protein–protein interaction between TLR4 and MD-2. Disrupting the large surface area of a protein–protein complex with a drug-like small molecule was formerly believed to be a daunting challenge.[1] However, in recent years the use of small-molecule inhibitors to probe protein–protein interactions has become a rapidly evolving field in chemical biology.[2–4] Toll-like receptor 4 (TLR4) is a membrane-spanning immune receptor that functions in complex with its accessory protein myeloid differentiation factor 2 (MD-2).[5] The TLR family is a group of type I integral membrane glycoproteins.[6] TLR4 detects lipopolysaccharide, LPS, a TLR4 agonist and component of gram-negative bacterial cell walls.[7] TLR4 signaling has been implicated in numerous disease states including acute sepsis and neuropathic pain.[8,9] The TLR4/MD-2 interaction is an attractive therapeutic target as it is essential to TLR4 signaling.[10] Furthermore, MD-2 interacts primarily with TLR4 among other homologous TLR family proteins,[6] therefore targeting the TLR4/MD-2 complex may provide a strategy to selectively regulate TLR4 signaling.

Computational techniques generally involve a balance between speed and accuracy. To predict the binding affinity of a compound for its protein target, most docking programs[11–17] employ two operations: “docking” and “scoring.” The first procedure performs a massive conformational search and generates multiple protein-ligand conformations, or “poses,” corresponding to probable ligand conformations within the protein binding pocket. In the second step, each of the docked poses is characterized by a measure of the affinity, calculated using a scoring function. Usually the top few binders are selected to quantify the binding properties of a compound. Docking algorithms usually provide a fast way to help enrich a library of compounds.[18–20] However, favor of high speed sometimes compromises prediction accuracy, generating high numbers of false positives and/or negatives. By contrast, molecular dynamics (MD) simulations can treat both proteins and ligands in a flexible manner, allowing for relaxation of the binding site around the ligand and even estimations of the effects of explicit water molecules. Moreover there are a number of MD-based techniques to access the binding free energy directly.[21,22] The most rigorous computational techniques are Thermodynamic Integration (TI) and Free Energy

Perturbation (FEP) methods, though the techniques are normally too computationally expensive for high-throughput calculations.

In order to improve affinity prediction accuracy without reducing screening speed, we developed a sequence of MM methods with implicit solvent models to evaluate binding free energies. Many recent investigations have revealed that similar methods, such as the MM/Poisson Boltzman-Surface Area (MM/PB-SA) approach proved to be highly capable of predicting the binding free energies.[23] To get through the bottleneck posed by the computationally demanding MD-based methods, we used a combination of the two: fast molecular docking for the generation of binding poses and MD simulations to rank the ligand poses according to their binding affinities implemented in QUANTUM.[23–26] To confine the amount of necessary calculations to a reasonable level, we performed extensive clustering of the ligand libraries to ensure the least possible computational work, while keeping as much of the full chemical diversity of the available library as possible. Finally, we conducted profiling of hits against a library of *ca*. 500 representative human proteins as a selectivity filter. As a proof of concept, screening was conducted against both TLR4 and MD-2 to validate our strategy and to maximize the potential of discovering a useful small molecule inhibitor. Compounds **1** and **2** were identified as potential TLR4- and MD-2-specific antagonists, respectively, with predicted K_d values less than 10 μM (Figure 1).

Because biophysical evaluation of TLR4 inhibitors is challenging due to the limited availability of TLR4 (recombinant expression of TLR4, an immune receptor that senses LPS, is not achievable in high-yielding bacterial systems), compounds **1** and **2** were tested in mammalian cells using a previously established TLR4 signaling assay.[27,28] LPS-induced TLR4 activation initiates the phosphoinositide 3-kinase (PI3K) cascade, triggering translocation of Akt1 to the plasma membrane in RAW264.7 cells.[29] Since Akt1 is immediately downstream to TLR4, activation of Akt has been widely used as an indication of TLR4 signaling. RAW cells transfected with Akt1-GFP[28] were activated with LPS in the presence or absence of **1** or **2** and translocation of Akt1-GFP to the plasma membrane was monitored. Compound **1** (2 μM) or compound **2** (200 nM) completely abolished LPS-induced activation of signaling (Figure 2), presumably due to the disruption of the critical TLR4/MD-2 complex formation. A chemotactic peptide C5a (25 ng/mL) that directly stimulates PI3K[30] rescued the Akt signaling, confirming that these cells retained normal functions and compound **1** and **2** are not toxic under these assay conditions.

A challenge when developing TLR4 inhibitors is engineering selectivity against other TLRs. We investigated the selectivity of **1** by measuring nitric oxide (NO) production in RAW264.7 cells. RAW cells express all TLRs and each specific TLR can be individually activated by treatment with a receptor-specific ligand.[31] Activation of TLRs results in downstream signaling and production of pro-inflammatory mediators such as nitric oxide (NO). Compound **1** (300 nM) inhibited TLR4-mediated NO production but showed negligible effects on the signaling of TLR3, TLR 2/6, TLR 2/1 and TLR7 (Figure 3). The excellent selectivity demonstrated by compound **1** demonstrates the ability of our screening process to identify a highly promising small molecule TLR-4 signaling inhibitor.

In summary, we have developed a new in silico high-throughput screening methodology, incorporating a MD optimization of the target protein-protein complex and selectivity profiling to improve binding affinity/specificity estimations without requiring significantly increased computational resources. We applied our methodology to identifying inhibitors of the protein protein interaction between TLR4 and MD-2, one inhibitor to target each protein. We demonstrated that these small molecule inhibitors were able to disrupt TLR4 signaling in vitro in RAW macrophage cells. Furthermore, we demonstrated that compound **1** was selective for TLR4 over other TLR family proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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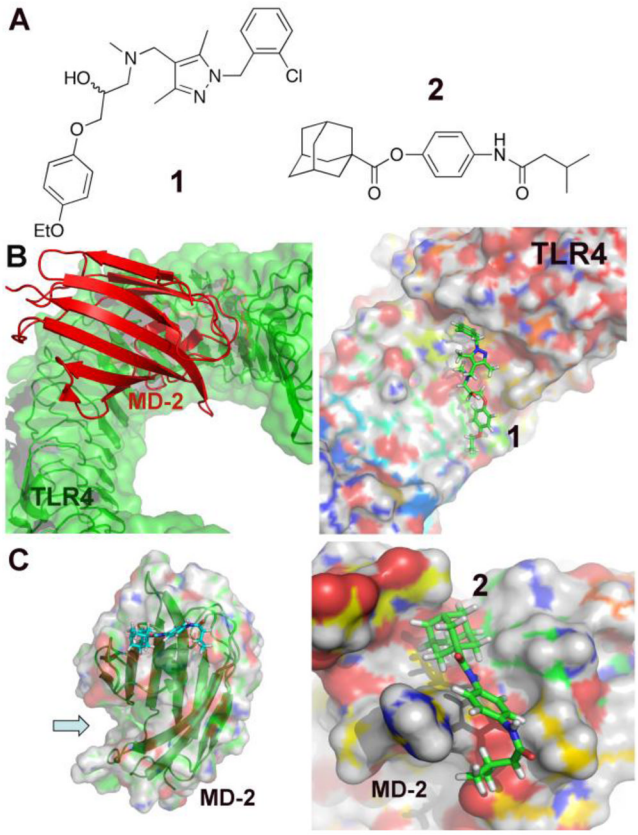

Figure 1.

Results of molecular docking to identify inhibitors that block the TLR4/MD-2 interface. (A) Chemical structures of identified TLR4/MD-2 complex inhibitors, **1** and **2**. (B) Results of the molecular-docking experiments of compound **1** binding to TLR4. The structure of the TLR4/MD-2 complex was taken from protein data bank (PDB ID 2Z65, resolution 2.70 Å). [5] (Left panel) The TLR4/MD-2 binding interface. (Right panel) Close-up view showing **1** (shown in stick representations) in the MD-2 binding site on the surface of TLR4. (C) Results of the molecular-docking experiments of compound **2** binding to MD-2. (Left panel) Global view of the **2**/human MD-2 complex, showing that **2** recognizes an allosteric site that is different from the LPS-binding site (arrow indicated) on the MD-2 surface. (Right panel) Close-up view showing that **2** recognizes the target pocket with high spatial complementarity.

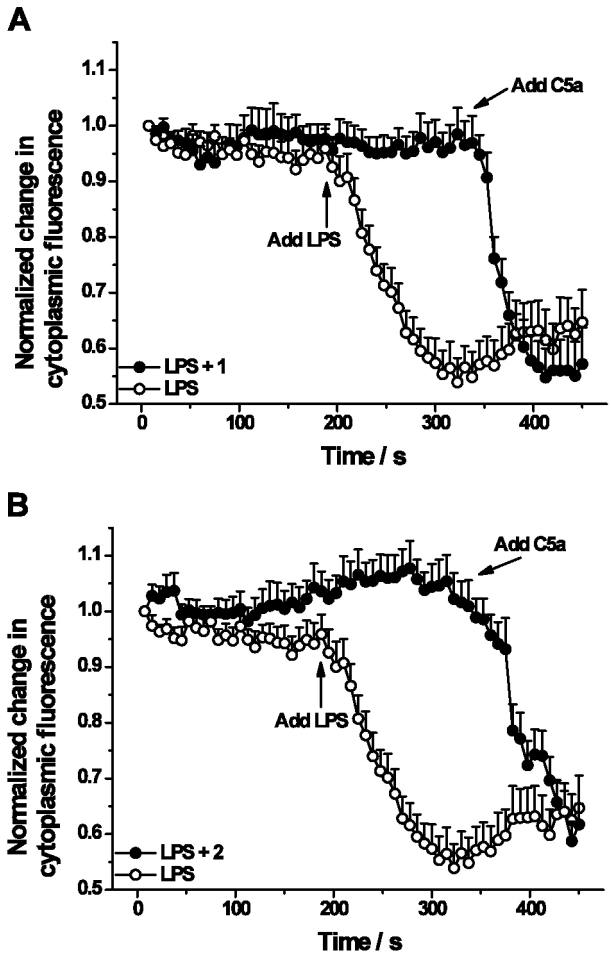

Figure 2.

Compounds **1** (panel A) and **2** (panel B) block LPS-induced TLR4 activation in macrophages. LPS was added to murine macrophages to a final concentration of 2 ng/mL, in the presence or absence of 2 μM **1** or 200 nM **2**. If no visual response was observed after an additional 2.5 minutes, 25 ng/mL C5a (an Akt signaling agonist via a non-TLR4 pathway) was added to confirm that the cells were still responsive.[30] Data are means from ten independent experiments; error bars show s.e.m.

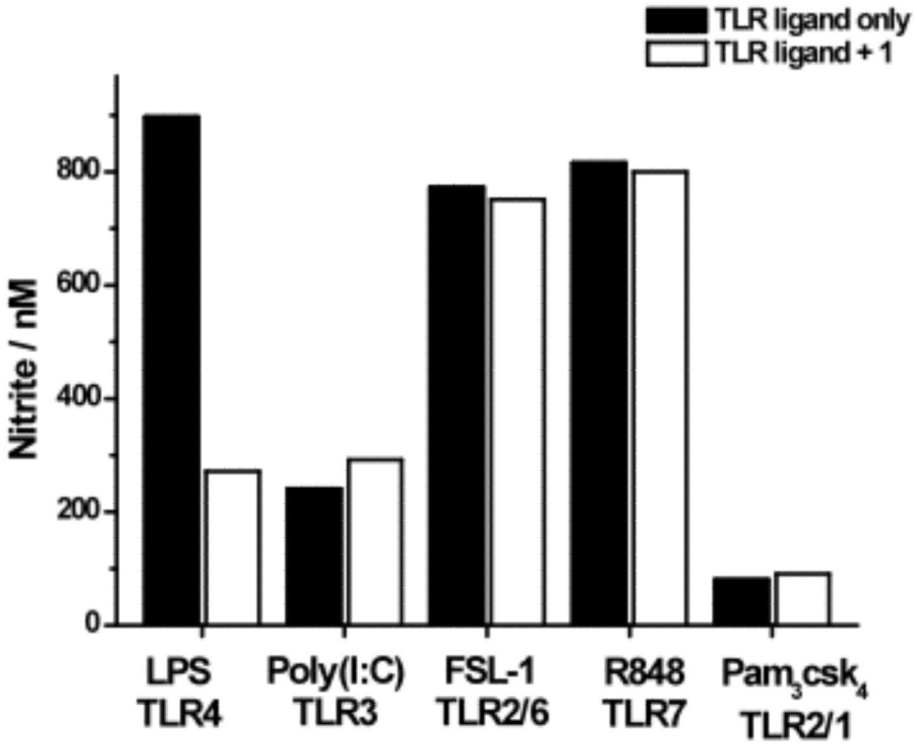

Figure 3.

Effect of **1** on TLR-ligand induced nitric oxide (NO) production in RAW264.7 cells. LPS (lipopolysaccharide), poly(I:C) (polyinosinic-polycytidylic acid), FSL-1 ((S,R)-(2,3-bispalmitoyloxypropyl)-Cys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe), R848 (4-amino-2-(ethoxymethyl)-α, α-dimethyl-1H-imidazo[4,5-c]quinoline- 1-ethanol) and Pam_3CSK_4 (N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2R,S)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine.3HCl) were used to selectively activate TLR4, TLR3, TLR2/6, TLR7 and TLR2/1 respectively. Compound 1 (300 nM) selectively inhibited the NO production induced by LPS, but not ligands of other TLRs.