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Discovery of Novel Ligands for TNF-*a* and TNF Receptor-1 through Structure-Based Virtual Screening and Biological Assay

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Abstract

Tumor necrosis factor a (TNF-a) is overexpressed in various diseases, and it has been a validated therapeutic target for autoimmune diseases. All therapeutics currently used to target TNF-a are biomacromolecules, and limited numbers of TNF-a chemical inhibitors have been reported, which makes the identification of small-molecule alternatives an urgent need. Recent studies have mainly focused on identifying small molecules that directly bind to TNF- α or TNF receptor-1 (TNFR1), inhibit the interaction between TNF-a and TNFR1, and/or regulate related signaling pathways. In this study, we combined in silico methods with biophysical and cell-based assays to identify novel antagonists that bind to TNF-a or TNFR1. Pharmacophore model filtering and molecular docking were applied to identify potential TNF- α antagonists. In regard to TNFR1, we constructed a threedimensional model of the TNF-a-TNFR1 complex and carried out molecular dynamics simulations to sample the conformations. The residues in TNF- α that have been reported to play important roles in the TNF-a-TNFR1 complex were removed to form a pocket for further virtual screening of TNFR1-binding ligands. We obtained 20 virtual hits and tested them using surface plasmon resonance-based assays, which resulted in one ligand that binds to TNFR1 and four ligands with different scaffolds that bind to TNF-a. T1 and R1, the two most active compounds with K_d values of 11 and 16 μ M for TNF- α and TNFR1, respectively, showed activities similar to those of known antagonists. Further cell-based assays also demonstrated that T1 and R1 have

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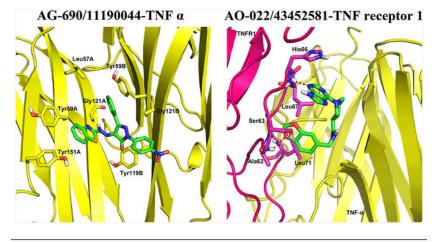
Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jcim.6b00672. TNF- α X-ray structures 1–15 (Table S1); 10 virtual hits tested by SPR assay for binding with TNF- α (Figure S1); 10 virtual hits tested by SPR assay for binding with TNFR1 (Figure S2); NMR and LC–MS spectra of the tested compounds (Figure S3); and measurement of affinity constants by SPR analysis (Figure S4) (PDF)

The authors declare no competing financial interest. The protein structure of the TNF- α -TNFR1 complex can be downloaded from our public Web site (http://www.cbligand.org/downloads/TNF_TNFR1.pdb).

similar activities compared to the known TNF- α antagonist C87. Our work has not only produced several TNF- α and TNFR1 antagonists with novel scaffolds for further structural optimization but also showcases the power of our in silico methods for TNF- α - and TNFR1-based drug discovery.

Graphical Abstract



INTRODUCTION

Tumor necrosis factor a (TNF-a) is an important cytokine with powerful proinflammatory and immunomodulatory effects.^{1,2} Overexpression of TNF-a is widely observed in HIV,³ asthma,⁴ and autoimmune diseases such as rheumatoid arthritis,⁵ Crohn's disease,⁶ and psoriasis.^{7,8} TNF-a has become a therapeutic target for autoimmune diseases with the successful launch of TNF-a antagonists, including infliximab, etanercept, adalimumab, certolizumab, and glolimumab.⁹ However, these biologic therapies exhibited inevitable weaknesses, such as risk of infection,¹⁰ high cost, and the requirement for intravenous injections. By contrast, small-molecule inhibitors are relatively cheaper and can be taken orally. Therefore, the identification of small molecules that can inhibit TNF-a-regulated pathways is a promising and current focus area.

Recent research has mainly focused on identifying small molecules that directly bind to TNF-*a* or TNF receptor-1 (TNFR1),^{11,12} inhibit the binding of TNF-*a* and TNFR1,^{13,14} and/or regulate related signal pathways.¹⁵ Figure 1 summarizes all of the published small-molecule inhibitors that bind to TNF-*a* or TNFR1 with $K_d < 50 \ \mu$ M, inhibit the interaction between TNF-*a* and TNFR1 with IC₅₀ < 50 \ \muM, or regulate TNF-*a*-mediated cellular effects with IC₅₀ < 50 \ \mu M.^{8,11,12,16} Some compounds with weaker activity were excluded, including several active herbal components previously published by our group.¹⁷ SPD-304, identified in 2005, was the first TNF-*a* antagonist, with a K_d value of 0.11 \ \muM.¹⁶ AP-906/41640035, a TNF-*a* inhibitor with an IC₅₀ of 100 \ \muM, was identified by molecular-docking-based virtual screening using the crystal structure of human TNF-*a* in complex with SPD-304 (Figure 1).¹² In regard to TNFR1, physcion-8-*O*- β -D-monoglucoside was the most active antagonist, with a K_d value of 0.376 \ \muM.¹¹ It can be concluded that only eight small-molecule inhibitors show moderate activity, and none of them have entered clinical

trials at present. Thus, there is an urgent need to identify novel inhibitors with the rapeutic potency for TNF-a-related disease.

After examining all of the available structures of TNF-*a* (summarized in Table S1 in the Supporting Information), we found no TNF-*a* trimer cocrystallized with small molecules, but we did find a TNF-*a* dimer structure binding with the small molecule SPD-304 (Protein Data Bank (PDB) code 2AZ5). SPD-304 slightly changed the angles of the two TNF-*a* monomers, promoting the dissociation of the third TNF-*a* monomer.¹⁸ The solution of the TNF-*a* dimer–SPD-304 structure provided a direction for in silico identification of TNF-*a* inhibitors that function by disrupting the trimer structure. However, no TNFR1 protein structures cocrystallized with small molecules have been solved to date, and thus, a related model needed to be constructed for in silico identify small molecules that inhibit the protein–protein interactions by mimicking or replacing the binding-site residues in one of the proteins.^{16,19,20} As the structures of TNFR1²¹ and the TNF-*a*–TNFR2 complex²² were available, superimposition and adjustment could be conducted to obtain the structure of the TNF-*a*–TNFR1 complex. Thus, we can mimic or replace the residues in TNF-*a* that bind to TNFR1 to identify TNFR1-binding ligands.

In the present study, we applied a virtual screening strategy combined with bioassay validations to identify novel TNF-*a*-and TNFR1-binding ligands. Virtual hits were subjected to a surface plasmon resonance (SPR)-based binding assay and a cell-based assay. To the best of our knowledge, this is the first study to identify TNFR1-binding ligands by an in silico method. Our strategy is likely to provide a new and efficient way for the identification of TNF-*a* and TNFR1 inverse agonists with therapeutic potential.

MATERIALS AND METHODS

Ligand Preparation.

The Specs database (2016) (http://www.Specs.net), a three-dimensional (3D) database with 213 293 compounds, was used as the ligand database. We first filtered it to eliminate metals or mixtures of isotopes using SYBYL-X 1.3.²³ After further removal of potentially promiscuous structures, also known as pan-assay interference compounds (PAINS), by our in-house tool PAINS-Remover (http://cbligand.org/PAINS/),²⁴ 191 361 compounds remained.

Structural Preparation of TNF-a-TNFR1 Complex and TNF-a.

To date, no NMR/X-ray crystal structures are available for the human TNF-*a*–TNFR1 complex. However, several studies have reported the structures of the TNF-*a*–TNFR2 complex and the extracellular domain of TNFR1 determined by single-crystal X-ray diffraction.^{21,22} The TNF-*a*–TNFR1 complex was obtained on the basis of the crystal structures of TNFR1 (PDB entry 1EXT, resolution 1.85 Å) and TNF-*a*–TNFR2 (PDB code 3ALQ, resolution 3 Å). The relative orientations of TNF-*a* and TNFR1 were based on the crystal structure of TNF-*a* bound with TNFR2. Superimposition and adjustment were applied using PyMOL (www.pymol.org) to obtain a TNF-*a*–TNFR1 complex. Briefly,

TNFR1 (sequence ID P19438–1) was superimposed onto the *a*-carbon (C*a*) atoms of TNFR2 (especially for M^{30} , H^{40} , C^{96} , C^{115} , and G^{126} ; sequence ID P20333–1). In addition, the crystal structure of human TNF-*a* with the antagonist SPD-304 (PDB code 2AZ5, resolution 2.1 Å) was used. The structures were downloaded from the PDB (http://www.rcsb.org/). SYBYL-X 1.3²³ was used to prepare the structure, including residual repair and energy minimization. The detailed parameters were described in our previous publications.^{23,25} ProSA-web Z-scores²⁶ and ProCheck Ramachandran plots²⁷ were used for structural stereochemical evaluation of the TNF-*a*–TNFR1 complex. The protein structure of the TNF-*a*–TNFR1 complex can be downloaded from our public Web site (http://www.cbligand.org/downloads/TNF_TNFR1.pdb).

Conformational Sampling and Secondary Energy Minimization for the TNF-*a*-TNFR1 Complex Model.

In order to select the most reasonable structure of the TNF-*a*–TNFR1 complex, we conducted a 50 ns molecular dynamics (MD) simulation to get an appropriate model. Determining the optimal protonation states for histidine residues is very important in MD studies.^{28,29} We applied VEGA ZZ 2.4.0³⁰ and PROPKA 3.1³¹ software to calculate the p*K* values of the protein. For the TNF-*a*–TNFR1 complex model, as the calculated p*K* values were lower than 7.40, no histidines were protonated. We set all of the histidine residues as "HIE", and then we carefully examined the residual environment of HIE and found that it is not necessary to change the HIE to HID. The Asp, Glu, Arg, and Lys residues were charged (Asp⁻, Glu⁻, Arg⁺, and Lys⁺) in our work.

We used the VMD program³² to embed the structure of the TNF-a-TNFR1 complex model into a pre-equilibrated and periodic structure of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC). The lipid molecules within 3 Å of the protein were eliminated and inserted into a water box with TIP3P water molecules. Then we eliminated the water molecules within 3 Å of the protein.

Briefly, the simulation systems for the TNF-*a*–TNFR1 complex contained the TNF-*a*–TNFR1 complex model, 67 133 atoms in total, 20 235 water molecules, etc. The box size was 86 Å \times 94 Å \times 90 Å. The first minimization, with 50 000 steps, was conducted with the protein fixed, while the second minimization, also with 50 000 steps, was performed with flexible protein. Then 1.0 ns of MD for heating and equilibration from 0 to 310 K was carried out.

On the basis of the last frame of the equilibration, the 50 ns MD simulation was performed using the AMBER package³³ with a ff99SB³⁴ force field for protein in explicit water. The particle mesh Ewald method with a nonbonded cutoff of 12 Å and a grid spacing of 1 Å per grid point in each dimension was applied to calculate the electrostatics. A cutoff (switching radius 10 Å, cutoff radius 12 Å) was applied to calculate the van der Waals energies. The temperature and pressure were kept constant using a Langevin thermostat (310 K) and a Langevin barostat (1 atm), respectively. The time step was set as 1 fs. The data were saved every 10 ps for analysis. We used VMD software to analyze the trajectories.

The conformation of the TNF-*a*–TNFR1 complex model with the lowest energy was subjected to secondary energy minimization using SYBYL-X 1.3.

Molecular Docking for the Studies of Ligand–TNF-*a* and Ligand–TNFR1 Interactions.

A series of dockings were conducted for the TNF-*a* and TNF-*a*–TNFR1 complex structures. The MOLCAD module in SYBYL-X 1.3 was used to define the binding pockets of TNF-*a* and the TNF-*a*–TNFR1 complex structure. Surflex-Dock, a docking program in SYBYL-X 1.3, was used to generate the detailed ligand–receptor interactions, in which the docking score was expressed as $-\log_{10}(K_d)$. We used the same docking parameters as described previously.^{35,36} Briefly, the starting conformation was set to 10, the maximum number of rotatable bonds to 100, each fragment's maximum conformation to 20, the Ångströms to expand search grid to 6, and each ligand's maximum number of poses to 100. The following flags were switched on: molecule fragmentation, postdock minimization, soft grid treatment, and predock minimization. The number of spins per alignment was set to 12, and the activated spin alignment method with density of search set to 3.0 was used. MMFF94 with MMFF94S charges were applied for preparing ligands. We selected the binding poses from the pose cluster on the basis of their frequencies and docking scores.

Pharmacophoric Filtering for TNF-a Antagonists.

Structural details from the known TNF-*a* inhibitor SPD-304 (Figure 1) were used to generate pharmacophoric filters. In this study, the GALAHAD program in SYBYL-X 1.3 was used to construct a three-point pharmacophore model, including three aromatic centers. We used the pharmacophore model to filter the Specs database for compounds satisfying specific geometric and physicochemical constraints using SYBYL-X 1.3. The detailed parameters are summarized below: population size, 20; keep best *N* models, 10; random number seed, 12 345; maximum generations, 90. Search options used default values.

Virtual Screening for Inhibitors of TNF-*a* and TNFR1.

After pharmacophoric filtering of the Specs database, we performed further virtual screening against TNF-a and TNFR1 by molecular docking, which was conducted using the Surflex-Dock program in SYBYL-X 1.3. The detailed docking parameters can be seen above.

Immobilization of TNFR1 and TNF-*a* on the Sensor Surface.

The immobilization of human TNFR1 and TNF-a on the sensor surface was carried out as described previously.^{11,17} Briefly, TNFR1 was diluted in 10 mM sodium acetate (pH 5.5) and immobilized by the amine coupling method on a CM5 sensor chip (GE Healthcare) according to the manufacturer's protocol. In addition, 50 μ g/mL TNF-a in 10 mM sodium acetate buffer (pH 5.0) was covalently immobilized onto the CM5 sensor chip using standard primary amine coupling procedure. SPR measurements were performed on a Biacore T200 system (GE Healthcare, Sweden).

SPR Screening and Affinity Analysis.

The specificity for TNFR1 binding was characterized using our previously identified TNFR1 antagonist physcion-8-O- β -D-monoglucoside (Figure 1), which was used as a positive

control.¹¹ Ten compounds were diluted in phosphate-buffered saline (PBS) running buffer containing 5% dimethyl sulfoxide (DMSO) to a concentration of 50 μ M. They were then injected to the TNFR1 sensor surface for 120 s at a flow rate of 30 μ L/min. Sensograms of these compounds were recorded and analyzed. AO-022/43452581 (R1) was selected for further affinity analysis. It was diluted in PBS running buffer containing 5% DMSO to concentrations ranging from 4 to 256 μ M. Analytes were injected through reference and active channels at a flow rate of 30 μ L/min. The association and dissociation time were both 120 s. The affinity fitting was carried out by Biacore T200 evaluation software using a steady-state affinity model to obtain the affinity constant K_d .

The specificity for TNF- α binding was validated using compound C87 as a positive control. ¹⁶ Ten compounds were diluted in PBS running buffer containing 5% DMSO at a concentration of 50 μ M. Gradient concentrations of components (2–128 μ M) dissolved in the running buffer were injected into the channel for 120 s, followed by dissociation for 120 s. The data were analyzed with the Biacore T200 evaluation software using a 1:1 binding model.

Assay for TNF-a-Induced L929 Cytotoxicity.

L929 cells were seeded in 96-well plates at 2.0×10^4 cells/well and cultured overnight. Different concentrations of chemicals (25–200 μ M) were mixed with 10 ng/mL TNF-*a* and 1 μ g/mL actinomycin D (AMD) and added to the cells. After 18 h of incubation, cell viability was assessed by microscope examination and the CCK-8 assay as described previously.¹⁷ The optical density (OD) was measured at 450 nm in a microplate reader (Synergy4, BioTek, USA). The percentage inhibition of cytotoxicity (%I) was calculated using the following formula: (OD_{actinomycinD+TNF-*a*+chemicals-OD_{actinomycinD+TNF-*a*)/(OD_{actinomycinD+TNF-*a*) × 100.}}}

$$\%I = \frac{\left(OD_{AMD+TFN-\alpha+chemical} - OD_{AMD+TFN-\alpha}\right)}{\left(OD_{AMD} - OD_{AMD+TFN-\alpha}\right)} \times 100\%$$

Predictions of Toxicity Properties.

Toxicity risks were predicted from precompiled fragment lists using DataWarrior.³⁷ The predicted toxicity properties consisted of mutagenicity, tumorigenicity, reproductive effect, and irritant effect.

RESULTS AND DISCUSSION

Structural Analysis of All Known Inhibitors.

Eight chemical structures and their bioactivity data (K_d and IC₅₀ > 50 μ M) against TNF- α , TNFR1, and/or TNF- α -TNFR1 interaction listed in Figure 1 were retrieved from the literature.^{8,11,12,16,38} As shown in Figure 1, these compounds have diverse structures and exhibit moderate activities with micromolar K_d and/or IC₅₀, indicating a limitation of TNF- α -related small-molecule inhibitors.

Key Residues in the Potential Binding Pocket of TNFR1.

The crystal structure of the TNF-*a*–TNFR1 complex is still unrevealed. On the basis of available crystal structures of TNFR1 (PDB code 1EXT, resolution 1.85 Å) and TNF-*a*–TNFR2 complex (PDB code 3ALQ, resolution 3 Å), we predicted the potential binding modes of TNFR1–TNF-*a* using alignment (Figure 2a). We selected TNFR1 with PDB code 1EXT (resolution 1.85 Å) because it has higher resolution than TNFR1 with PDB code 1NCF (resolution 2.25 Å). A group of residues in TNF-*a*, including Ala84B, Val85B, Ser86B, Tyr87B, Gln88B, and Thr89B, were selected as residues that binding to TNFR1. Several other residues around these six residues were used to form a pocket for molecular docking to screen TNFR1-binding ligands. The pocket was formed by TNFR1 and monomer B of TNF-*a* (Figure 2b). We chose these six residues for two reasons. First, our structural model indicated that these residues in TNF-*a* are directly involved in the binding with TNFR1 (Figure 2a). Second, they had been reported to play important roles in the interactions of TNF-*a* with TNFR1.^{39,40} We tried to mimic these six residues to identify antagonists that bind to TNFR1.

Convergence Parameters of MD.

In order to validate the TNF-a-TNFR1 complex model, we performed a 50 ns MD simulation for this system. Figure 3 indicates the root-mean-square deviation (RMSD) of the TNF-a-TNFR1 complex. This system reached equilibrium after 5 ns with an average RMSD of 4.0 Å, which revealed that the TNF-a-TNFR1 complex model is a stable protein.

Pharmacophore-Model-Based Filtering.

The GALAHAD program in SYBYL-X 1.3 was used to construct a pharmacophore model. The TNF-*a*-related pharmacophore model was based on the TNF-*a* inhibitor SPD-304. For clarity, we highlighted the pharmacophores at the binding site. Shown in Figure 4 is SPD-304 in complex with TNF-*a*, where three pharmacophores in the first model are composed of three aromatic centers (A1–A3). This model was applied to filter the prefiltered Specs database. An optimized 3D chemical compound library with 6601 compounds was obtained after filtering.

Molecular-Docking-Based Virtual Screening.

After removal of PAINS, a virtual screening was carried out using the optimized 3D chemical compound library. The TNF-a-TNFR1 complex (Figure 2a) with the lowest energy during our MD simulations was used to perform the virtual screening against the Specs database. In addition, we also used the TNF-a structure (PDB code 2AZ5, resolution 2.1 Å) to conduct a virtual screening of the 6601 compounds prefiltered by the TNF-a antagonist's pharmacophore model.

Novel TNF-a-Binding Ligands from in Silico Screening.

We measured the TNF-*a*-binding affinities (K_d) of 10 compounds from the Specs database by an SPR-based binding assay. The structures of the 10 virtual hits are shown in Figure S1 in the Supporting Information. The previously identified TNF-*a* antagonist C87 was used as a positive control,¹⁶ which yielded a K_d value for TNF-*a* of 6 μ M (Figure 5a,b). Among the

10 commercial compounds, four yielded K_d values against TNF-*a* of 11, 58, 72, and 113 μM (Table 1). T1 emerged as the best-binding ligand tested. As shown in Figure 5, T1 exhibited activity similar to that of the positive control (C87).

Novel TNFR1-Binding Ligands from in Silico Screening.

To evaluate the model for identifying novel small molecules that bind to TNFR1, we examined a panel of 10 compounds from the Specs database. The structures of these 10 virtual hits can be seen in Figure S2. Our previously identified TNFR1 antagonist physcion-8-O- β -D-monoglucoside was used as a positive control.¹¹ Among the 10 compounds, R1 yielded a K_d value for TNFR1 of 16 μ M (Figure 6). Compared with the known inhibitors of TNFR1 in Figure 1, R1 exhibited a novel scaffold, and it might be used as a novel chemical probe for future studies.

Compounds Inhibit TNF-a-Induced L929 Cell Death.

We next evaluated the efficacies of all of the hits. The ¹H NMR and MS spectra of the five hits and C87 are shown in Figure S3. As expected, C87 as a positive control significantly reduced L929 cell death induced by TNF-a (Figure 7a,b).^{11,14,16,17} R1, T1, and T3 showed dose-dependent inhibition against TNF-a-mediated cytotoxicity on L929 cells within the range of 25–200 μ M (Figure 7). Strikingly, the activity values of all three compounds were comparable to or better than that of the positive control C87. However, T2 and T4 displayed no activity. Of note, although the positive control C87 was tested to be active, our tested value (IC₅₀ \approx 200 μ M) differed a lot from the reported value (IC₅₀ \approx 10 μ M). There may be several reasons for this phenomenon. First, although our TNF-a-mediated cytotoxicity was induced by 10 ng/mL TNF-a and 1 μ g/mL AMD as in previous studies,^{11,14,17} the literaturereported L929 cytotoxicity was generated by 1 ng/mL TNF-a and 1 μ g/mL AMD. Therefore, the different degree of cytotoxicity might lead to different activity of C87. In addition, the different seeding densities $(2 \times 10^4 \text{ cells/well vs } 1 \times 10^4 \text{ cells/well})$, different vendors of TNF-a, different passage of L929 cells, and different experimental staffs might also have caused the above phenomenon. Nevertheless, C87 can still validate the reliability of our assay. Since the activity values of compounds could be affected by multiple factors, the activity of C87 can serve as an internal reference for comparing the activities of other hits. The strongest activity values of T1, T3, and R1 remain to be further investigated.

Insight into the Binding Mode of T1 against TNF-a.

The detailed interactions of T1 with TNF-*a* were modeled. T1 and SPD-304 share high similarities in their interactions with TNF-*a* in their corresponding bound states. As shown in Figure 8, both of them have three hydrophobic interactions with the same residues, likely contributing to the observation that the binding affinities of T1 ($K_d = 11 \mu M$) and SPD-304 ($K_d = 5.36 \mu M$) are on the same order of magnitude. A group of residues consisting of Leu57A, Gly121A, and Tyr59B form a hydro-phobic interaction with TNF-*a* antagonists. Tyr59A and Tyr151A, Tyr119B, and Gly121B form another two groups (Figure 8).

Insight into the Binding Mode of R1 against TNFR1.

We also modeled the detailed interactions between R1 and TNFR1 (Figure 9). Compared with the known binding residues for TNF-a, R1 shares high similarities in the interaction with TNFR1. As shown in Figure 9, R1 and the six residues in TNF-a (Ala84B, Val85B, Ser86B, Tyr87B, Gln88B, and Thr89B) both form strong hydrogen bonds with Ala62 and His66 in TNFR1. The K_d value for binding of TNF-a to TNFR1 is 1.76 nM,³⁹ whereas the K_d value for R1 was 16 μ M in our binding assay (Figure 6). The mechanisms for the weaker binding activity of R1 still need further investigation. Taken together, our modeling studies demonstrate that site-specific hydrophobic interactions and hydrogen bonds contribute to the binding of our screened ligands against TNF-a and TNFR1. These interaction patterns may facilitate further screening for more potent antagonists of TNF-a or TNFR1.

Toxicity Properties of Biological Hits.

We predicted our biological hits' toxicity properties, including mutagenicity, tumorigenicity, reproductive effect, and irritant effect, using DataWarrior.³⁷ As shown in Table 2, only R1 did not show any toxicity properties, suggesting that R1 may be a promising lead compound. As for the other four compounds, there existed several kinds of toxicity properties, including mutagenicity, tumorigenicity, and reproductive effect. However, further chemical modifications might weaken or eliminate these properties. The predictions of the toxicity properties can provide references for identifying compounds with therapeutic potential.

CONCLUSIONS

In this study, we combined in silico strategies with biophysical and cell-based assays to identify novel antagonists that bind to TNF- α or TNFR1 and obtained five novel scaffolds with moderate binding affinities against the target proteins. The two most active compounds, T1 and R1, showed low-micromolar binding affinities to TNF- α and TNFR1, respectively, which are similar to those of known antagonists (Figure 1). Cell-based assays demonstrated that T1 and R1 also showed activities similar to that of C87 and T3 displayed better activity than C87. Analyses of the binding modes of T1 and R1 with TNF- α and TNFR1, respectively, has provided useful structural information for improved in silico screening and chemical modification. The summary of the structures and activities of all known inhibitors will also give us a better understanding of the structural features and may provide useful information for the identification of new skeletons. Our new approach based on pharmacophores and molecular docking will promote future virtual screening of novel TNF- α or TNFR1 antagonists with therapeutic potential.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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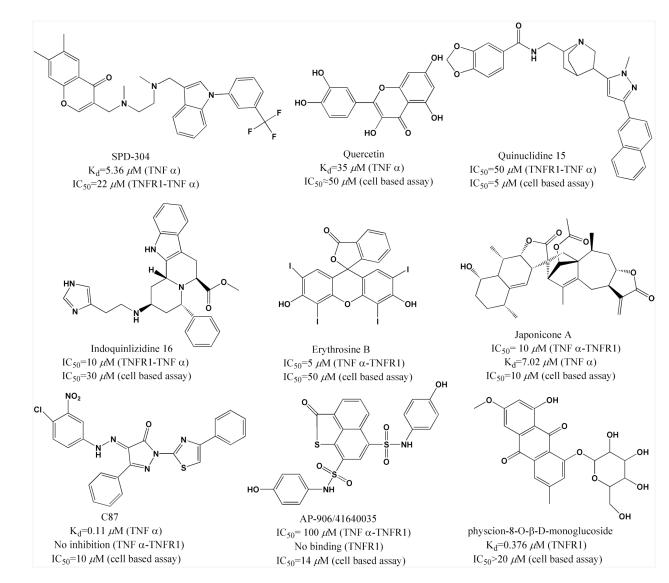


Figure 1.

All of the published small molecules that directly bind to TNF-*a* or TNFR1 or inhibit the binding of TNF-*a* and TNFR1 (K_d and IC₅₀ > 50 μ M). K_d (TNFR1) and K_d (TNF-*a*) are equilibrium dissociation constants showing compound binding affinities to TNFR1 and TNF-*a*, respectively, as determined by SPR analysis. IC₅₀ (TNF-*a*–TNFR1) is the inhibitor activity tested with competitive inhibition of TNFR1 binding to immobilized TNF-*a* as determined by enzyme-linked immunosorbent assay (ELISA). IC₅₀ (TNF-*a*–TNFR1) is the inhibitor activity tested with competitive inhibition of TNF-*a* binding to immobilized TNFR1 as determined by ELISA. IC₅₀ (cell-based assay) indicates inhibition of TNF-*a*-and TNFR1-related apoptosis or signaling changes.

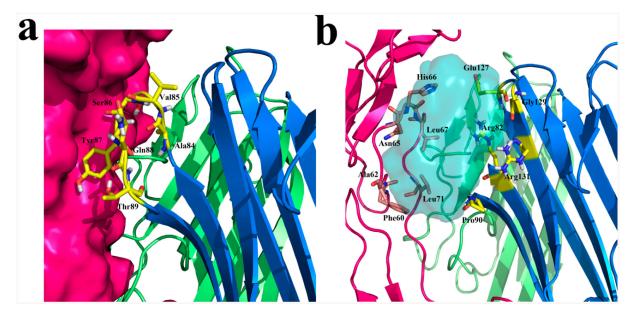


Figure 2.

(a) Residues in TNF-*a* (blue) that bind to TNFR1 (hot pink). (b) Binding pocket (light blue) of TNFR1 (hot pink). The pocket is formed by TNFR1 and a monomer of TNF-*a* (blue). The important residues are shown in sticks, including residues in TNFR1 (Phe60, Ala62, Asn65, His66, Leu67, and Leu71) and TNF-*a* (Arg82, Pro90, Gly129, and Arg131).

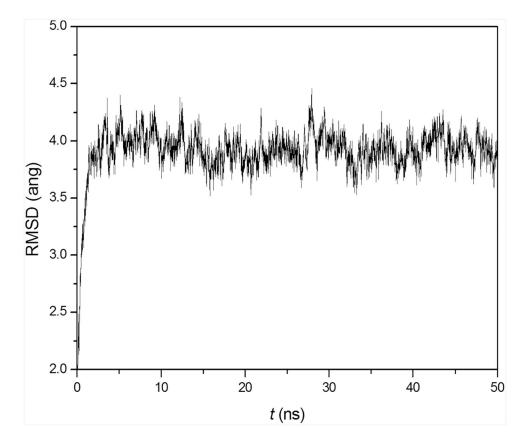


Figure 3.

Time evolution of the RMSD of the TNF-a-TNFR1 complex during the 50 ns MD simulation.

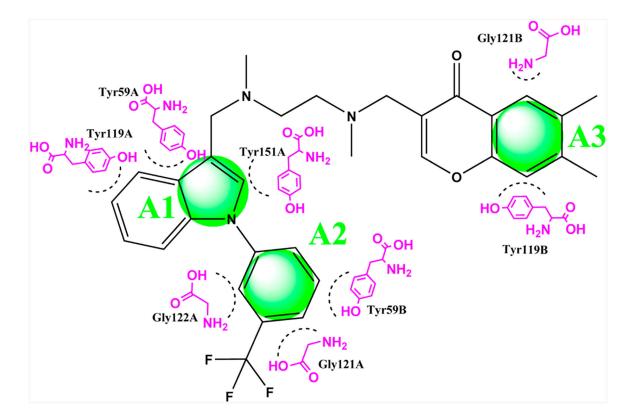


Figure 4.

Two-dimensional pharmacophore model H3 showed three aromatic centers (A). The model was based on SPD-304, which was crystallized with TNF-a (PDB code: 2AZ5).

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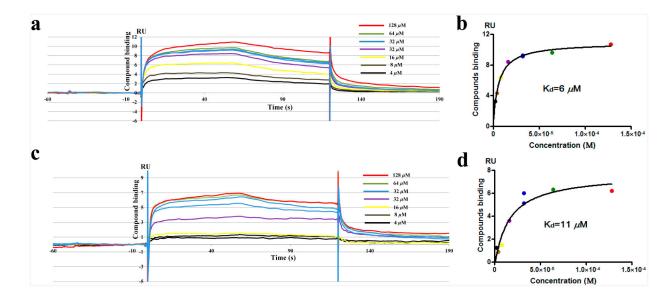


Figure 5.

Measurement of affinity constants by SPR analysis. (a, c) Sensograms recorded after injection of different concentrations of the positive control (C87) and T1, respectively. (b, d) Affinity constant (K_d) values of the positive control and T1, respectively. The K_d values were calculated by global fitting using a steady-state affinity model. The data shown are representatives of three independent experiments.

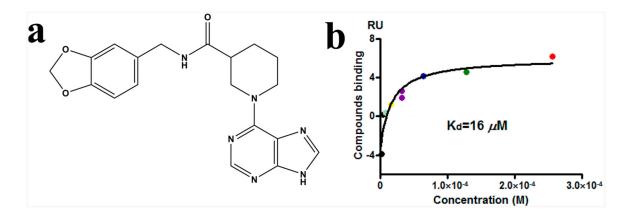


Figure 6.

(a) Chemical structure of R1 from in silico screening. (b) Affinity constant (K_d) value for R1. The value was calculated by global fitting using a steady-state affinity model. The data in (b) are representative of three independent experiments.

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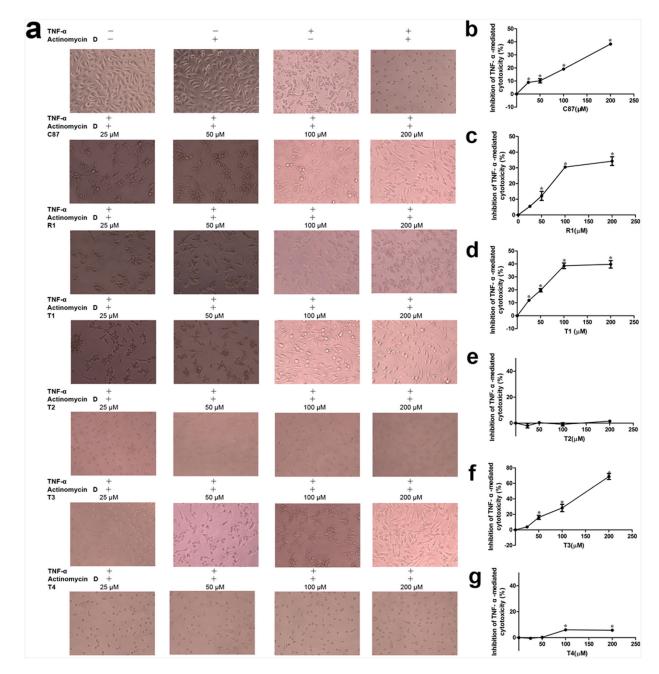


Figure 7.

Inhibition activities of C87, R1, T1, T2, T3, and T4 against TNF-*a*-mediated cytotoxicity on L929 cells. L929 cells were treated for 18 h with 10 ng/mL TNF-*a* and 1 μ g/mL actinomycin D in the presence of the indicated concentrations of C87, R1, T1, T2, T3, and T4. C87 was used as a positive control. (a) Cell viability was examined under microscope (×200). (b–g) TNF-*a*-mediated cytotoxicities on L929 cells were measured with the CCK-8 assay. Data were obtained from three independent experiments performed in triplicate and are presented as mean ± standard error of the mean. *, *p* < 0.05 vs TNF-*a* only.

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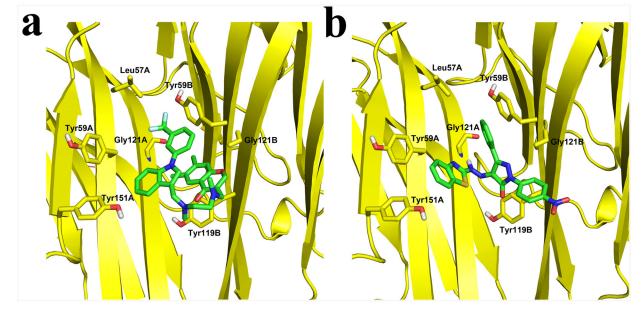


Figure 8.

Detailed binding modes of antagonists with TNF-*a*, including (a) SPD-304 and (b) T1. The three same hydrophobic interactions are formed by both SPD-304 and T1.

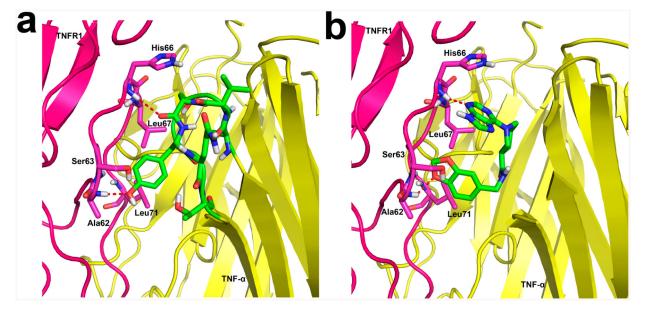


Figure 9.

Detailed binding modes of antagonists with TNFR1, including (a) residues in TNF- α in green (Ala84, Val85, Ser86, Tyr87, Gln88 and Thr89) and (b) R1. Two residues in TNFR1 (Ala62 and His66) form strong hydrogen bonds with the antagonists.

Table 1.

Chemical Structures, Properties, and TNF-*a*-Binding Values of the Hits from Virtual Screening

Compound ID Structure	Structure	SPECS ID	AlogP MW	МW	Kd (µM) TNF-a.
11	of a	AG-690/11190044 5.5	5.5	442.45	=
T 2	TS+6	AG-690/15438091 5.11	5.11	479.53	58
T3		AG-205/37136129 5.81	5.81	562.50	72
T4	876 7	AG-667/37281063 4.81	4.81	422.50	113

Table 2.

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Predicted Toxicity Properties of the Biological Hits

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Compound ID	Structure	SPECS ID	Target protein	Mutagenicity	Tumorigenicity	Target protein Mutagenicity Tumorigenicity Reproductive effect Irritant effect	Irritant effect
II	8-40 2-40	AG-690/11190044 TNF-a	TNF-a	none	none	high	none
RI	-0-B	AO-022/43452581	TNFR1	none	none	none	none
T2	78+S	AG-690/15438091	TNF-a	high	high	none	none
Т3	off	AG-205/37136129	TNF-α	none	none	low	none
T4	876	AG-667/37281063	TNF-a	high	high	none	none