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Structure-Based Discovery of Natural Product-Like TNF-α Inhibitors

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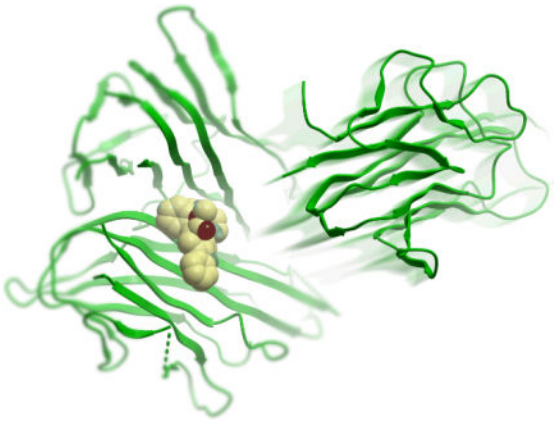
Abstract



Two natural product-like inhibitors of TNF-α have been identified using structure-based virtual screening. These compounds represent only the third and fourth examples of direct target of TNF-α by a small molecule and display comparable potency to the strongest TNF-α inhibitor reported to date.

Keywords

inhibitors; tumor necrosis factor; virtual screening; natural products; drug discovery

Tumor necrosis factor α (TNF-α) is a multifunctional cytokine that acts as a central biological mediator for critical immune functions, including inflammation, infection, and antitumour responses.[1] Dysregulation of TNF-α has been implicated in cases of tumorigenesis, diabetes, and especially in autoinflammatory diseases such as rheumatoid arthritis, psoriatic arthritis and Crohn's disease.[2] The synthetic antibodies etanercept, infliximab, and adalimumab approved for the treatment of inflammatory diseases bind to TNF-α directly, preventing its association with the tumor necrosis factor receptor (TNFR).[3] However, their potential to cause serious side effects such as eliciting an autoimmune anti-antibody response or the weakening of the body's immune defenses to opportunistic infections, has stimulated the development of alternative small molecule-based therapies to TNF-α inhibition.[4] Most such small molecule inhibitors reported in the literature target TNF-α indirectly.[5–8]

To our knowledge, the only small molecules capable of antagonizing TNF-α directly are the polysulfonated naphthylurea suramin and its analogues,[9] and the indole-linked chromone designated SPD304 (Figure 1).[10] Unfortunately, the low potency and poor selectivity of suramin coupled with its tendency to cause adverse side effects renders it unsuitable for anti-TNF-α therapies.[11] Furthermore, SPD304 containing the toxic 3-alkylindole moiety was found to be metabolized by cytochrome P450 enzymes *via* a similar dehydrogenation pathway as the potent pneumotoxin 3-methylindole, producing reactive electrophilic iminium species capable of conjugating protein and/or DNA targets.[12] Therefore, the

development of relatively less toxic small molecule inhibitors of TNF-α for therapeutic applications remains a highly desirable goal.

Natural products (NPs) have been refined over evolutionary time scales for optimal interactions with biomolecules. Not surprisingly, NPs have represented a cornerstone of pharmaceutical research, as they offer a diverse range of chemical scaffolds, bioactive substructures, and potentially lower toxicity profiles.[13] Historically, many approved drugs have been NPs, while numerous others were derived from or inspired by a NP template.[14] Encouraged by these ideas, and by the relative dearth of potent and non-toxic small molecule inhibitors directly targeting TNF-α, we sought to apply high-throughput, ligand docking-based virtual screening methods to identify TNF-α inhibitors from a natural product chemical libraries. We used the X-ray co-crystal structure of TNF-α dimer with SPD304 (PDB code: 2AZ5)[10] as the molecular model for our investigation.

Like most protein-protein interfaces, the binding pocket of the TNF-α dimer is relatively large and featureless, and lacks clearly-defined binding crevices or mechanism-based contacts.[15] The binding site is mostly hydrophobic, consisting primarily of glycine, leucine and tyrosine residues. Not unexpectedly, the binding interaction of small molecule SPD304 to TNF-α has been described to be predominantly hydrophobic and shape-driven.[10] Small-molecule inhibitors of TNF-α should thus be relatively hydrophobic and large enough to contact both subunits of the TNF-α dimer simultaneously, in order to prevent the binding of the third subunit forming the biologically active trimer complex.

Over 20,000 compounds from a chemical library of natural product/natural product-like structures[16] were screened *in silico*. The continuously flexible ligands were docked to a grid representation of the receptor and assigned a score reflecting the quality of the complex according to the internal coordinate mechanics (ICM) method [ICM-Pro 3.6-1d molecular docking software (Molsoft)].[18] The highest-scoring 16 compounds from the virtual screening results were tested in a preliminary ELISA to assess their ability to inhibit the binding of TNF-α to TNFR-1. Two chemically distinct structures, the pyrazole-linked quinuclidine **1** and the indolo[2,3-*a*]quinolizidine **2**, emerged as the top candidates (Figure 1). The binding poses of these two compounds overlap well with the crystallographic pose of SPD304 to TNF-α (Figure 2). Like SPD304, compounds **1** and **2** are large enough to contact residues from both subunits of the TNF-α dimer, thus occupying and blocking the binding site for the third TNF-α subunit.

In the top-scoring binding mode of **1** to the TNF-α dimer, the pyrazole-linked quinuclidine substructure occupies the hydrophobic binding pocket, and the dioxolane oxygen atom of **1** forms a hydrogen bond with the backbone amino group of Gly121 of TNF-a subunit B (Figure 2a). Compound **2** is not predicted to occupy the region of space close to Gly121 of subunit B, but instead forms a hydrogen bond with the side-chain hydroxyl group of Tyr151 of subunit B through its imidazole functionality (Figure 2b). Common features of the predicted binding modes of **1**, **2** and SPD304 are the extended hydrophobic ring systems that are in contact with the β-strand (Leu120–Gly121–Gly122) of TNF-α subunit A, and the presence of polar functional groups orientated away from the binding pocket and exposed to the aqueous environment. Interestingly, whereas the indole substructures of **2** and SPD304

(Figure 2c) are located in a similar region of space, their orientations with respect to the β-strand of subunit A are different. The lack of salt bridges or hydrogen bonding networks in our models of **1** and **2** with TNF-α is consistent with previous findings that the interaction between the small molecule SPD304 and TNF-α is primarily hydrophobic and shape-driven.[10] The calculated binding scores of −34.7 and −36.4 for **1** and **2** respectively reflect a strong interaction between the compounds and the dimer complex. As a reference, we calculated the binding score of SPD304 to be −32.9. The predicted binding coordinates of SPD304 in the binding pocket are within 1.0 Å root-mean-square deviation of the reported values based on the protein X-ray crystal structure.[10]

The quinuclidine core of **1** is present in a variety of natural products, such as the antimalarial cinchona alkaloids.[18] Natural products containing the indolo[2,3-*a*]quinolizidine scaffold of **2** include the alkaloids geissoschizine, deplancheine, corynantheidine, and yohimbane.[19] Waldmann and co-workers employed a biology-orientated synthetic approach to generate indolo[2,3-*a*]quinolizidine inhibitors of mycobacterial protein tyrosine phosphatase B.[20] To the best of our knowledge, no TNF-α-binding activity nor any other biological activity of **1** or **2** has been reported in the literature.

To validate the results of our molecular modeling, we performed dose-response experiments with compounds **1** and **2** to determine their half-maximal inhibitory concentration (IC_{50}) values against the TNF-α–TNFR-1 interaction using an ELISA (Figure 3). Encouragingly, indoloquinolizidine **2** (IC_{50} = *ca*. 10 μM) was found to be more active than SPD304, the most potent small molecule TNF-α inhibitor reported to date (IC_{50} = 22 μM by a comparable ELISA).[10,21] Quinuclidine **1** was moderately active against TNF-α with an IC_{50} value of approximately 50 μM.

We next investigated the ability of compounds **1** and **2** to inhibit TNF-α signaling in human cells. TNF-α solutions pre-incubated with the test compound were added to HepG2 cells, which were stably transfected with the NF-κB–luciferase gene. The inhibition of TNF-α-induced NF-κB signaling by the test compound was detected by monitoring the reduction in the luciferase activity of the cell lysates (Figure 4). Surprisingly, indoloquinolizidine **2** (IC_{50} > 30 μM) was found to be less active than quinuclidine **1** (IC_{50} = *ca*. 5 μM) in the cellular luciferase assay, despite showing greater potency in the cell-free ELISA. Notably, **2** exhibited a similar IC_{50} value similar to that of to SPD304 (IC_{50} = *ca*. 3 μM) as measured by our system, although it was less potent than SPD304 at higher concentrations. We hypothesize that the markedly reduced activity of **2** in cell culture could be because of its low bioavailability resulting from either poor cellular uptake or metabolic degradation of **2**.[22]

In conclusion, we have discovered two small molecules TNF-α inhibitors from a natural-product and natural-product-like chemical library using structure-based design. The identification of quinuclidine **1** and quinolizine **2** represents, to the best of our knowledge, only the third and fourth examples of the direct targeting of TNF-α by a small molecule. Importantly, indoloquinolizidine **2** (IC_{50} = *ca*. 10 μM) was found to be more potent against TNF-α in the ELISA compared to SPD304, the strongest small molecule TNF-α inhibitor reported to date. Quinuclidine **1** (IC_{50} = *ca*. 5 μM) displayed a comparable activity to

SPD304 (IC_{50} = *ca*. 3 μM) against cellular TNF-α induced NF-κB signaling. We are currently conducting computer-based hit-to-lead optimization to generate further analogues for *in vitro* testing.

Acknowledgments

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21. In a side-by-side experiment, we measured the inhibition of TNFR-1 binding by SPD304 (10 μM) to be 34%.
22. Using Molsoft's molecular property prediction tool, we calculated 2 to be less "drug-like" than 1. See the Supporting Information for details.

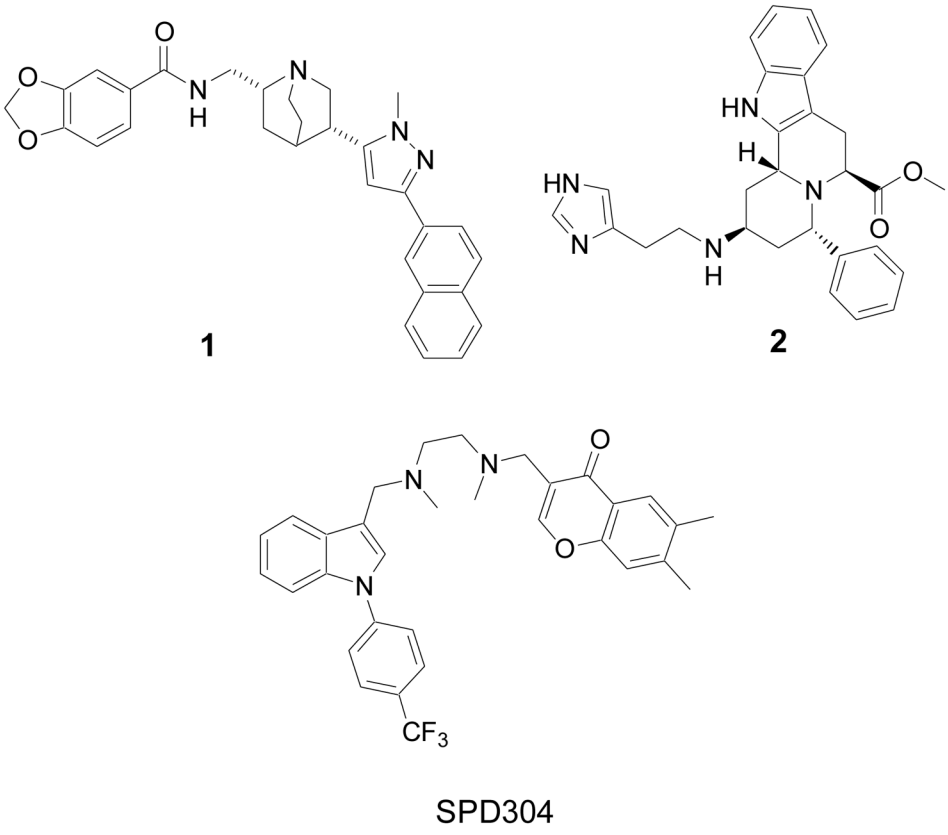

Figure 1.
Chemical structures of small molecule TNF-α inhibitors quinuclidine **1**, indoloquinolizidine **2** and SPD304.

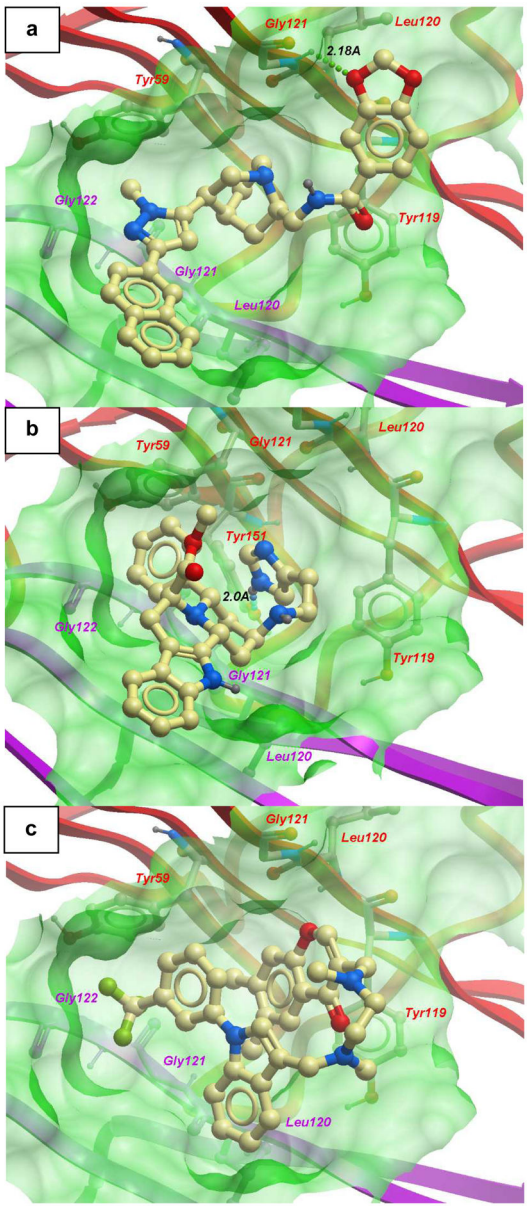

Figure 2.

Low-energy binding conformations of a) **1**, b) **2** and c) SPD304 bound to TNF-α dimer generated by virtual ligand docking. The two subunits of the TNF-α dimer are depicted in ribbon form and are colored in contrasted purple (subunit A) and red (subunit B). The small molecules are depicted as a ball-and-stick model showing carbon (yellow), hydrogen (grey), oxygen (red), nitrogen (blue), and fluoride (green) atoms. Hydrogen bonds are depicted as dotted lines. The binding pocket of the TNF-α dimer is represented as a translucent green surface.

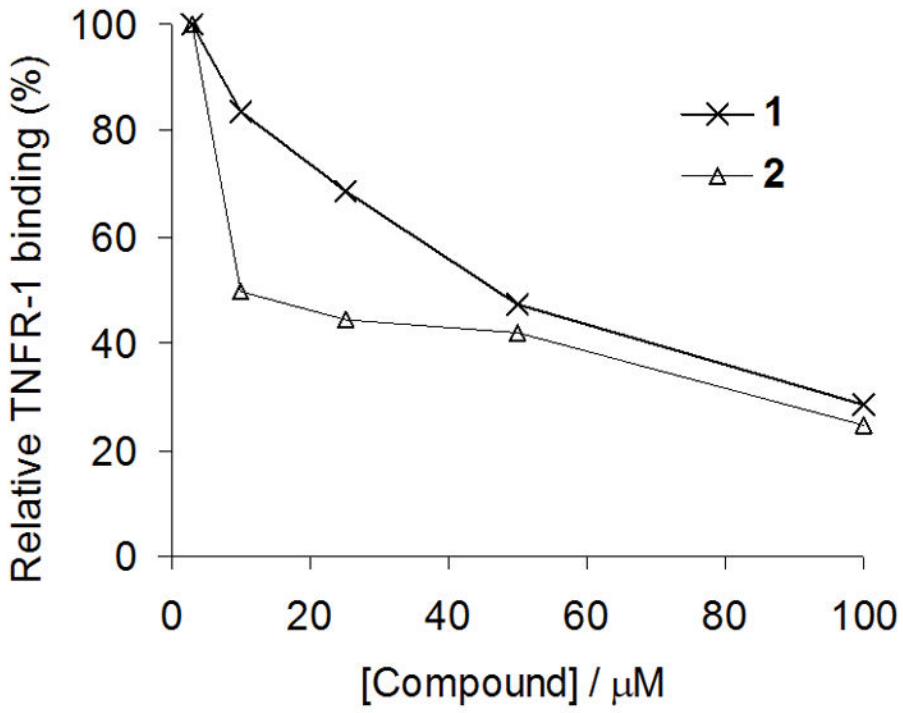

Figure 3.

Compound inhibition of TNFR-1 binding to immobilized TNF-α (ELISA). Microtitre plates coated with TNF-α were incubated with TNFR-1 together with **1** or **2** at the indicated concentrations. TNFR-1 binding was detected using anti-TNFR antibody and horseradish peroxidase-conjugated secondary antibody. Approximate IC_{50} values; **1**: 10 μM, **2**: 50 μM.

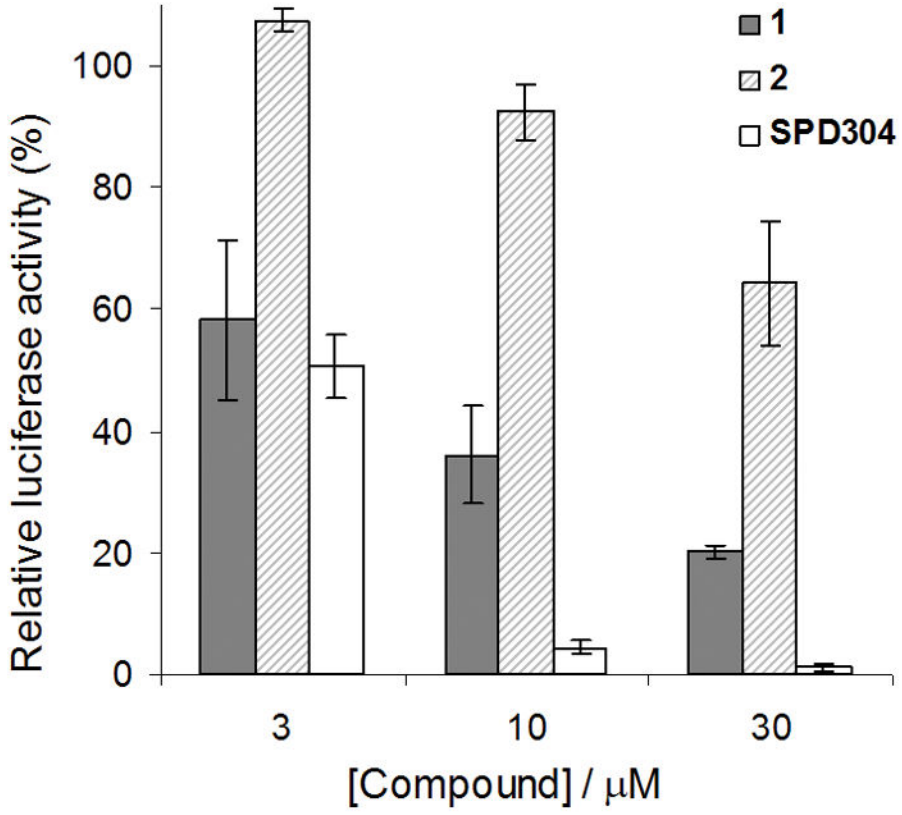

Figure 4.

Compound inhibition of cellular TNF-α-induced NF-κB activity. HepG2 cells stably transfected with the NF-κB–luciferase gene were stimulated with TNF-α pre-incubated with the indicated concentrations of **1**, **2**, or SPD304. Cell lysates were analyzed for luciferase activity to determine the extent of NF-κB inhibition. Approximate IC_{50} values; **1**: 5 μM, **2**: >30 μM, SPD304: 3 μM.