

Structure-Based Discovery of Natural-Product-like TNF- α Inhibitors**

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Angewandte
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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 antitumor 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 through a dehydrogenation pathway similar to that of the potent pneumotoxin 3-methylindole, producing reactive electrophilic iminium species capable of reacting with protein and DNA targets.^[12] Therefore, the development of relatively less toxic small-

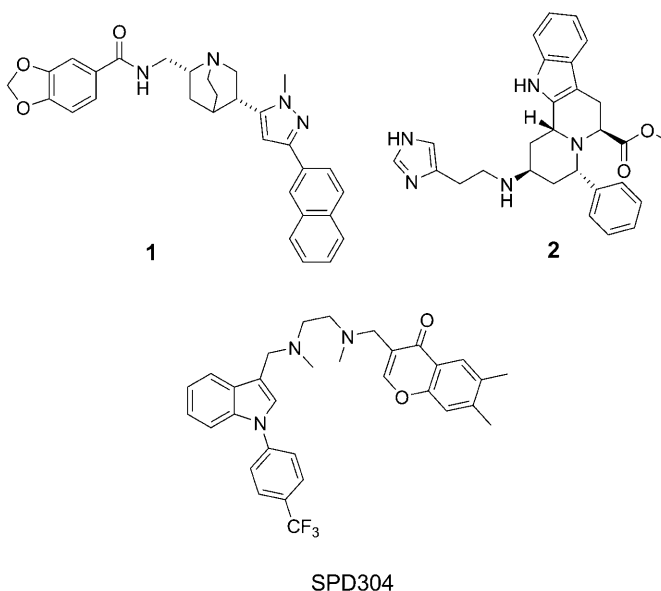


Figure 1. Chemical structures of small-molecule TNF- α inhibitors quinclidine **1**, indoloquinolizidine **2**, and SPD304.

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, whereas numerous others were derived from or inspired by a NP template.^[14] Encouraged by these ideas, and by the relative dearth of potent and nontoxic 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 natural-product chemical libraries. We used the X-ray cocrystal structure of a 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 the small molecule SPD304 to TNF- α has been described to be predominantly hydrophobic and shape-driven.^[10] Small-molecule inhibitors of TNF- α should be therefore relatively hydrophobic and large enough to interact with both subunits of the TNF- α dimer simultaneously to prevent the binding of the third subunit, which forms the biologically active trimer complex.

Over 20000 compounds from a chemical library of natural-product and 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-

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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 interact with the residues from both subunits of the TNF- α dimer, thereby 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- α 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 hydroxy 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 oriented 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 derived from 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-oriented 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** ($IC_{50} \approx 10$ μ M) was found to be more active than SPD304, the most potent small-molecule TNF- α inhibitor reported to date ($IC_{50} = 22$ μ M by a comparable ELISA).^[10,21] Quinuclidine **1**

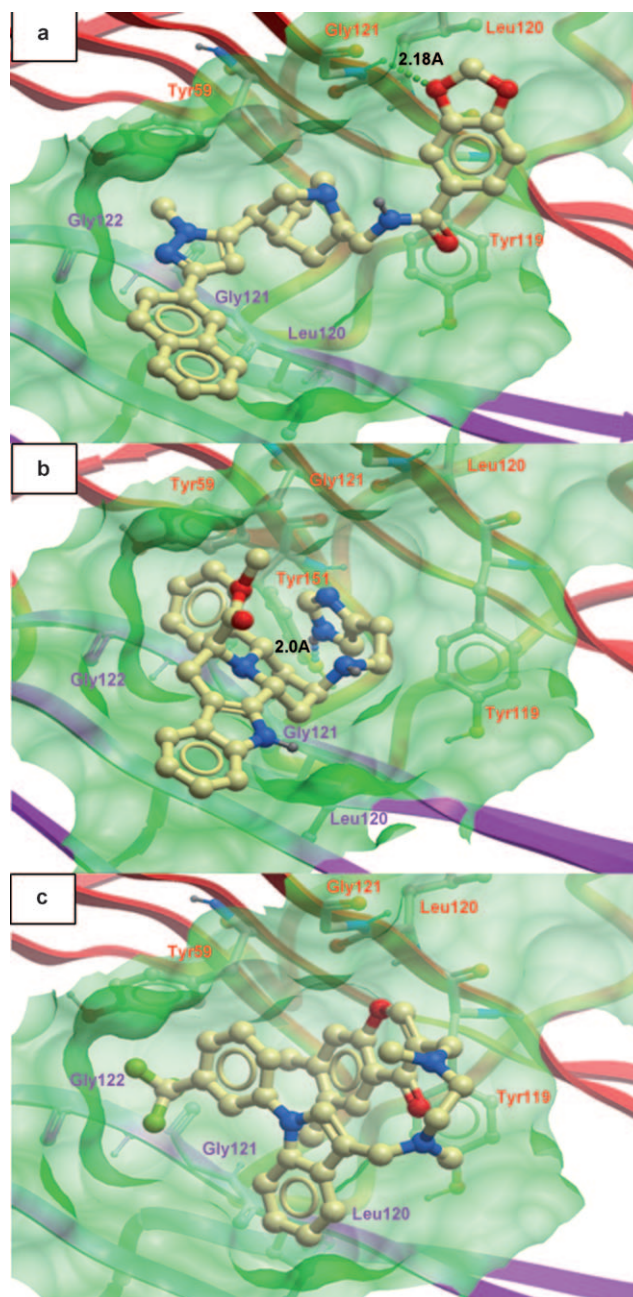


Figure 2. Low-energy binding conformations of a) **1**, b) **2**, and c) SPD304 bound to the TNF- α dimer generated by virtual ligand docking. The two subunits of the TNF- α dimer are depicted in ribbon form and are colored purple (subunit A) and red (subunit B). The small molecules are depicted as ball-and-stick models 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.

was moderately active against TNF- α with an IC_{50} value of approximately 50 μ M.

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- κ B-luciferase gene.

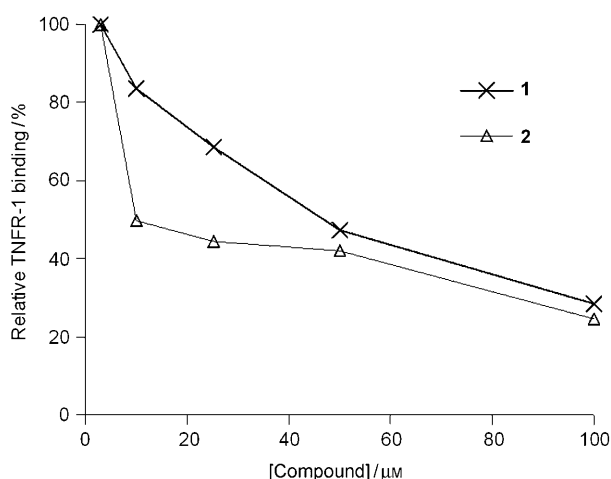


Figure 3. Compound inhibition of TNFR-1 binding to immobilized TNF- α (ELISA). Microtiter plates coated with TNF- α were incubated with TNFR-1 together with **1** or **2** at the indicated concentrations. TNFR-1 binding was detected using an anti-TNFR antibody and horseradish peroxidase conjugated secondary antibody. Approximate IC_{50} values: **1**: 10 μ M and **2**: 5 μ M.

The inhibition of TNF- α -induced NF- κ B signaling by the test compound was detected by monitoring the reduction in the luciferase activity of the cell lysates (Figure 4). Surprisingly, indoloquinolizidine **2** ($IC_{50} > 30 \mu$ M) was found to be less active than quinuclidine **1** ($IC_{50} \approx 5 \mu$ M) in the cellular luciferase assay, despite showing greater potency in the cell-free ELISA. Notably, **2** exhibited a IC_{50} value similar to that of SPD304 ($IC_{50} \approx 3 \mu$ M) as measured by our system, although it was less potent than SPD304 at higher concentrations. We

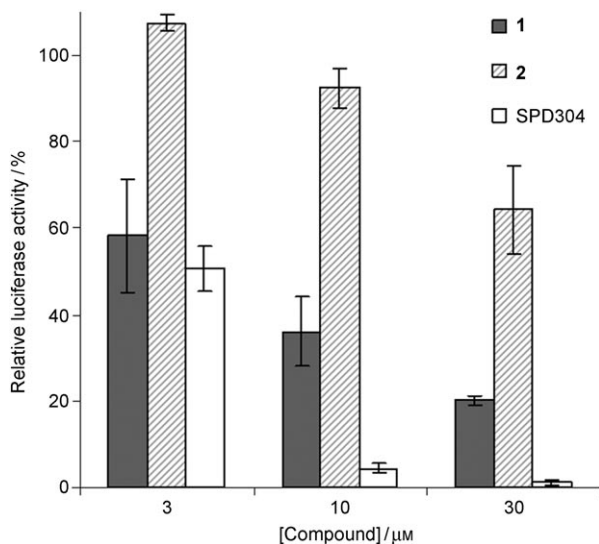


Figure 4. Compound inhibition of cellular TNF- α -induced NF- κ B activity. HepG2 cells stably transfected with the NF- κ B-luciferase gene were stimulated with TNF- α pre-incubated with the indicated concentrations of **1**, **2**, or SPD304. Cell lysates were analyzed for luciferase activity to determine the extent of NF- κ B inhibition. Approximate IC_{50} values: **1**: 5 μ M, **2**: $> 30 \mu$ M, and SPD304: 3 μ M.

hypothesize that the markedly reduced activity of **2** in the 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-molecule TNF- α inhibitors from a natural-product and natural-product-like chemical libraries using structure-based design. The identification of quinuclidine **1** and quinolizidine **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** ($IC_{50} \approx 10 \mu$ M) 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** ($IC_{50} \approx 5 \mu$ M) displayed activity comparable to that of SPD304 ($IC_{50} \approx 3 \mu$ M) against cellular TNF- α induced NF- κ B signaling. We are currently conducting computer-based hit-to-lead optimization to generate additional analogues for in vitro testing.

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 - [22] Using Molsoft's molecular property prediction tool, we calculated **2** to be less “drug-like” than **1**. See the Supporting Information for details.
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