A Novel Small-molecule Tumor Necrosis Factor α Inhibitor Attenuates Inflammation in a Hepatitis Mouse Model*

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Background: Most commercial TNFα inhibitors are biomacromolecules.

Results: A lead compound named C87 was identified using computer-aided drug design and could attenuate murine acute hepatitis.

Conclusion: C87 was one of the first effective small-molecule inhibitors of TNFα identified to date.

Significance: The study highlights the effectiveness of combining virtual screening with functional assays for developing novel small-molecule TNFα inhibitors.

Overexpression of tumor necrosis factor α (TNFα) is a hallmark of many inflammatory diseases, including rheumatoid arthritis, inflammatory bowel disease, and septic shock and hepatitis, making it a potential therapeutic target for clinical interventions. To explore chemical inhibitors against TNFα activity, we applied computer-aided drug design combined with in vitro and cell-based assays and identified a lead chemical compound, (E)-4-(2-(4-chloro-3-nitrophenyl) (named as C87 thereafter), which directly binds to TNFα, potently inhibits TNFα-induced cytotoxicity (IC₅₀ = 8.73 μM) and effectively blocks TNFα-triggered signaling activities. Furthermore, by using a murine acute hepatitis model, we showed that C87 attenuates TNFα-induced inflammation, thereby markedly reducing injuries to the liver and improving animal survival. Thus, our results lead to a novel and highly specific small-molecule TNFα inhibitor, which can be potentially used to treat TNFα-mediated inflammatory diseases.

TNFα, mainly secreted by active monocytes/macrophages, is known as a pleiotropic cytokine that plays crucial roles in host immune system (1). TNFα-mediated signaling has been shown to modulate a variety of cellular functions including survival, proliferation, and cell death (2, 3). TNFα signals are mediated through two distinct receptors, TNFR1 (p55) and TNFR2 (p75). It is generally believed that TNFR1 mediates most of TNFα activities because it is constitutively expressed in most tissues. In contrast, TNFR2 is typically found in immune cells (4–6). An elevated serum level of TNFα can cause many inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, septic shock, multiple sclerosis, and hepatitis (7, 8).

Because of the profound implications of TNFα in inflammatory diseases, direct inhibition of TNFα has become a major strategy in the treatment of those diseases. Significant advances have been made in the development of biological agents targeting TNFα and its signaling components. There are several well known commercial TNFα inhibitors such as infliximab, adalimumab, and etanercept, all of which are TNFα antibodies or TNFRI-Fc chimeras and function to prevent TNFα from binding to its receptor. To date, those biomacromolecular agents have been proved to be effective in the treatment of inflammatory bowel disease and rheumatoid arthritis due to their unique superiorities such as high specificity (9–13). However, several severe limitations such as poor stability, cost-ineffective commercial-scale production and exclusion from blood/brain barrier have also emerged. Instead, small-molecule chemical compounds have been appreciated as appropriate alternatives for overcoming most disadvantages associated with macromolecular inhibitors. Furthermore, they offer additional clinical benefits such as simpler preparation for oral medicine (14, 15).

Thus far, by the use of computer-aided drug design and cell-based assays in vitro, several selective small-molecule antagonists of TNFα activity have been identified. They include broad spectrum inhibitors targeting the key molecules of the intracellular TNFα pathway, functionally uncharacterized inhibitors of TNFα expression, inhibitors of the processing enzyme TNFα-

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¶The abbreviations used are: TNFR, TNF receptor; hTNFα, human TNFα; SPR, surface plasmon resonance.
In Vitro and in Vivo Activity of Novel TNFα Inhibitor

converting enzyme, and molecules that directly bind to TNFR or prevent TNFα-TNFR interactions (11, 15). Although these small-molecule inhibitors are capable of blocking the biological activity of TNFα in vitro, few have been shown to abrogate or reduce TNFα-induced inflammatory responses in vivo and exhibit high IC_{50} and severe side effects. Also, none of the small-molecular inhibitors have been reported to successfully block interaction of TNFα with TNFR through direct binding to TNFα in vivo. Thus, development of small molecules for TNFα therapy remains a major challenge. Currently, the crystal structure of TNFβ-TNFR1 complex, which clearly reveals the clusters of residues within the interface, is still the only available and reliable template for analysis of the critical interactive sites of TNFR1 and its ligand. As a result, the structure has been used as a basis for the rational design of TNF inhibitors. Because both TNFα and TNFβ belong to the TNF superfamily and share strikingly similar three-dimensional structures despite the low sequence homology (16–18), it is thus possible to use the computer-aided drug design platform to design small molecules that block the interaction between TNFα and its cognate receptors. Indeed, Takasaki et al. (19) successfully developed short peptides that mimic the loop 1/domain 3 of the TNFR1 and are capable of blocking TNFα signaling. However, these short peptides, despite their activities, have limitations in commercial production and clinic use for TNFα inhibition in vivo (19, 20).

In this study, we identified a novel TNFα chemical inhibitor, named C87, through virtual screening in combination with in vitro and in vivo assays. We found that C87 directly binds to TNFα, potently inhibits TNFα-induced cytotoxicity and effectively blocks TNFα-triggered signaling activities. More importantly, C87 attenuates TNFα-induced inflammation in vivo, thereby markedly reducing injuries to the liver and improving the survival of the animals.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Chemical Compounds—**L929, HL60, K562, and Jurkat cells were purchased from ATCC and maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone). Compounds were purchased from Specs Company and Sigma-Aldrich.

**Cytokines and Reagents—**Recombinant human and mouse TNFα were from Miltenyi, and LPS and d-GalN were purchased from Sigma. Recombinant human TNFβ, anti-TNFβ antibody, and anti-TNFα antibody were from Peprotech, and anti-Fas IgM antibody was from MBL (SY-001). The following antibodies were used for Western blotting immunofluorescence, and all of them were from Cell Signaling Technology: caspase-8 (4790), cleaved caspase-8 (8592), caspase-3 (9662), cleaved caspase-3 (9661), JNK (9258), p-JNK (4668), IκBα (4814), and GAPDH (2118). HRP-conjugated or AF-488-conjugated secondary antibodies were from Invitrogen. MTT, actinomycin D, dimethyl sulfoxide, LPS, and d-GalN were purchased from Sigma, and Eantercept (Enbrel) from Pfizer. The real-time PCR primers for mouse KC were 5’-TCGTCTTTTC-ATATGTATGGTACA-3’ and 5’-CGGACGAGGACGAGGC-3’. The primers for mouse TNFs were 5’-CATC- TCTCAAAAATTCGAGTGACA-3’ and 5’-TGGGAGTTAGAACGATCACCC-3’. The real-time PCR primers for mouse IL-1α were 5’-TCTCAGATTCAACTGTGTTCGTG-3’ and 5’-AGAAAAATGAGGGTCTCTCTGACTA-3’. The real-time PCR primers for mouse MIP-2 were 5’-CCCTGGTCTGAATAACCTCATC-3’ and 5’-AAC TCT CAG ACA GCC AGG CAC ATC-3’. The real-time PCR primers for mouse IFN-1 were 5’-GTTGTGCACTAAGACTCCCTG-3’ and 5’-GTGGCCGGCTAATCCTCC-3’. The primers for the mouse housekeeping gene GAPDH were 5’-TTTACAC- CATGGAAGGC-3’ and 5’-GGCATGAGCTGTGCTAGA-3’ and were used as a control.

**Virtual Screening—**Computer modeling was performed by using Silicon Graphics O2 work station. The compound library including ~90,000 small molecular compounds was switched to a 3D-mol2 file by using SYBYL software (version 6.7). The crystal structure of the TNFβ-TNFR1 complex (Protein Data Bank code 1TNR) from Brookhaven Protein Data Bank was used to study the sequences of TNFR1 that could potentially interact with TNFα. The key binding sequence was chosen as docking template.

**Cytotoxicity Measurement—**The inhibition of the cytotoxic effect of human TNFα by Specs compounds was measured in L929 cell line. Briefly, L929 cells were seeded at a density of 10^4 cells/well in a 96-well plate and incubated for 24 h in RPMI 1640 with 10% FBS at 37 °C. Different compounds were added to the cells with 1 μg/ml actinomycin D and 1 ng/ml TNFα and then incubated at 37 °C for 20 h before analysis. Cell survival was measured using the MTT method.

**SPR Assay—**Human TNFα was purified with an anti-E tag affinity chromatography (GE Healthcare) using the RPAS Purification Module (Amersham Biosciences), and its biological activities were determined by using cytotoxicity assay with commercial available human TNFα (hTNFα) as control (Miltenyi Biotec). Surface plasmon resonance (SPR) assays were carried out on a BIAcore 3000 instrument (Biacore, Inc.). Briefly, purified hTNFα was dissolved at 320 μg/ml in PBS, pH 7.0, and coupled to a carboxyl surface (HR-5 chip from HRBio, Inc.) by following the manufacturer’s instructions. For the equilibrium-binding experiments, the flow rate was set to 10 μl min^-1, and 50 μl of the compound sample was injected. For the kinetic experiments, the flow rate was also 10 μl min^-1, and 50 μl was injected for kinetic assay. A global analysis using the BIAEVALUATION software (version 3.0; Biacore, Inc.) and Igor (version 6.0, WaveMetrics, Inc.) was used to determine the kinetic curves. Briefly, the integrated rate equation describing a 1:1 Langmuir interaction was fit simultaneously to the entire concentration range for each compound. This fit yielded the association rate (K_a), the dissociation rate (K_d), and the dissociation constant (assuming the relationship K_D = K_d/K_a). The goodness of fit was determined by the χ^2 values, as well as the magnitude and distribution of the residuals.

**TNFα-TNFR Binding Assay—**High protein-binding 96-well plates (Dynex Technologies) were coated with recombinant hTNFα (100 ng per well) (Miltenyi Biotec) for overnight in 50 mM Na_2CO_3/NaHCO_3, pH 9.6. Before use, wells were blocked with 10% FBS in PBS for 1 h at room temperature. His-tag-labeled recombinant hTNFR1 or hTNFR2 (R&D Systems) in PBS containing 0.1% BSA was added at a final concentration of 1.5 μg/ml. C87 was serially diluted in dimethyl sulfoxide and...
mixed with hTNFR1 or hTNFR2 before addition into the plates. After the addition of C87 and purified receptors, plates were incubated for 2 h at room temperature and then washed five times with PBST. Anti-His antibody conjugated with HRP was added and incubated for 1 h. After several washes, the absorbance was measured at the wavelength of 450 nm by following the protocol provided by the TMB assay kit (Tiangen Biotec).

**Murine Hepatitis Model**—BALB/c mice (8–12 weeks old) were obtained from the Laboratory Animal Center of Institute of Hematology and Blood Diseases Hospital (Chinese Academy

**FIGURE 1. Screening for hTNFα chemical inhibitors.** Compounds from Spec library were named C1 to C102. The capacity of preventing hTNFα-induced cell death in L929 cells were measured in three different concentrations for each compound. One dot represents one experiment, and each treatment has been repeated independently for three times. Chemical compound C87 was found able to significantly prevent cell death induced by TNFα.

**FIGURE 2. Structure activity relationship analysis of C87.** A, chemical structure of C87. B, C87 prevented hTNFα-induced cell death under different concentrations. The IC50 of C87 was determined as 8.73 μM. C, inhibition of hTNFα-induced cell death by C87 and its analogs. The inhibition was measured in different concentrations (0.4, 4, and 20 μM). Survive rates were determined as average ± S.D. Each experiment has been repeated three times.
of Medical Sciences). All procedures involving mice were approved and monitored by the Research Ethics Committee of the Institute. Before LPS/GalN challenge (LPS (50 µg/kg) and D-GalN (1.2g/kg)), mice were injected (intraperitoneally) with C87 (12.5 mg/kg), Enbrel (4 mg/kg), or vehicle, respectively, at 1, 8, and 16 h. Blood was collected with retro-orbital sampling.

Activities of alanine transaminase and aspartate transaminase were detected by following the manufacturer’s instructions (Nanjing Jiancheng Bioengineering Institute). Liver tissues were collected and fixed in 10% formalin, and sections were stained with hematoxylin and eosin.

**Statistical Analysis**—Statistical analyses were conducted with Student’s paired t test using GraphPad Prism (San Diego, CA). Data were shown as means ± S.D.

### RESULTS

**Screening for TNFα Chemical Inhibitors**—The availability of crystal structure of the protein complex has provided essential information for inhibitor screening in the past (21, 22). But the crystal structure of the TNFα-TNFRI complex has not been determined to date. However, TNFα shares the same receptor and has high structural similarities to TNFβ. In this study, we chose a seven-amino acid peptide of the loop 2/domain 2 of TNFRI (RKEMGQV, amino acids 77–83), which has been identified as one of those three potential key sites for TNF/TNFRI interactions (19, 20), as the docking template for virtual screening of chemical compounds that target TNFα. Of ~90,000 compounds examined, 965 were identified to closely mimic the spatial structure of the initial docking template, and 102 compounds (designated as C1–C102) were chosen as lead compounds based on their binding energy and potential for future drug development. We applied three different concen-

### TABLE 1

| The structure and molecule formula of C87 analogs and A8 |
|------------------|------------------|------------------|------------------|------------------|
| **structure**    | **molecule formula** |
| A1               | C<sub>15</sub>H<sub>16</sub>N<sub>4</sub>S       |
| A2               | C<sub>15</sub>H<sub>16</sub>N<sub>4</sub>S       |
| A3               | C<sub>15</sub>H<sub>16</sub>N<sub>4</sub>S       |
| A4               | C<sub>15</sub>H<sub>16</sub>N<sub>4</sub>S       |

**A**  
Control  
TNFα  
A8  
C87  
Anti-TNFα

**B**  
Actinomycin D  
TNFα  
A8  
C87  
Anti-TNFα

**C**  
ADR  
VP16  
TNFα

**D**  
Anti-Fas IgM  
PBS

**E**  
Actinomycin D  
Control  
TNFβ  
A8  
C87  
Anti-TNFβ

**FIGURE 3.** C87 potently inhibited hTNFα-induced cell death. A, morphology of L929 cells after treated with hTNFα alone, hTNFα with A8, hTNFα with C87 and hTNFα with an anti-TNFα antibody. B, apoptotic assay of L929 cells with the above-mentioned treatments. C, survival of L929 cells after treated with ADR, VP16, hTNFα, and different concentrations of C87. D, survival of Jurkat cells after treated with anti-Fas IgM and different concentration of C87. E, C87 failed to prevent anti-Fas induced cell death in Jurkat cells. Results were shown as average ± S.D. Each experiment has been repeated three times.

**TABLE 1**

The structure and molecule formula of C87 analogs and A8

|   |   |   |   |
|---|---|---|---|
|   | A1 | A2 | A3 | A4 |
|   | C<sub>15</sub>H<sub>16</sub>N<sub>4</sub>S | C<sub>15</sub>H<sub>16</sub>N<sub>4</sub>S | C<sub>15</sub>H<sub>16</sub>N<sub>4</sub>S | C<sub>15</sub>H<sub>16</sub>N<sub>4</sub>S |
|   | A5 | A6 | A7 | A8 |
|   | C<sub>15</sub>H<sub>16</sub>N<sub>4</sub>S | C<sub>15</sub>H<sub>16</sub>N<sub>4</sub>S | C<sub>15</sub>H<sub>16</sub>N<sub>4</sub>S | C<sub>15</sub>H<sub>16</sub>N<sub>4</sub>S |
trations of the chemical compounds during initial tests to examine their ability to inhibit TNF\(\alpha\)/H9251-induced cytotoxicity in L929 cell line (Fig. 1). Two compounds, C34 and C87, were found to potently inhibit the activities of TNF\(\alpha\)/H9251 (Fig. 1). In further study, we found that C34 had poor solubility even in dimethyl sulfoxide and thus aborted further functional validations of this compound. Instead, C87, also called (E)-4-(2-(4-chloro-3-nitrophenyl)), exhibited good solubility and consistent dose-dependent functions and therefore was selected for further characterization and functional validation in vitro and in vivo.

Structure-Activity Relationship Analysis—The structure of C87 is shown in Fig. 2A. Experiments with L929 cells showed that the IC\(_{50}\) of C87 for TNF\(\alpha\)-mediated cytotoxicity was 8.73 \(\mu\)M (Fig. 2B), indicating high efficacy and potential as a novel TNF\(\alpha\) inhibitor. To determine the structure-activity relationship of C87, a series of 1,3-disubstituted-4-arylhydrazono-pyrazol-5-ones (A1–A7) as analogues of the lead inhibitor C87, together with (E)-2-hydroxy-5-((4-(N-pyridin-2-ylsulfamoyl)phenyl)diazenyl) benzoic acid (A8), which has a different structural feature, were selected to compare their potential to inhibit TNF\(\alpha\)-induced cytotoxicity in L929 cells (Table 1 and Fig. 2C). We found that all compounds except A1 and A3 showed dose-dependent inhibition of TNF\(\alpha\)-induced cytotoxicity to various degrees. It is worth noting that compounds with the E configuration of the C=N double bond appeared more potent than those with Z configuration. For instance, compounds A2, A4, and C87, which all contain the C=N double bond in the E configuration, elevated the survival rate to ~30, 45, and 70%, respectively, at the concentration of 20 \(\mu\)M. In addition, compounds with the nitro group at 3\(-\) position and chlorine at 4\(-\) position on the benzene ring of the arylhydrazono group (A4 and C87) showed stronger inhibitory activity, and the compound (C87) with phenyl group at the 3\(-\) position of pyrazolone ring was more effective than that with methyl group (A4). Together, despite the inhibitory effects of several compounds (e.g. A2 and A4) at higher concentrations, none of them were as effective as C87 (Fig. 2C).

**C87 Potently and Specifically Inhibits TNF\(\alpha\)-induced Cell Death**—We next examined the efficacy and specificity of C87. As expected, a TNF\(\alpha\)-neutralizing antibody markedly reduced L929 cell death induced by TNF\(\alpha\) (Fig. 3A). Strikingly, C87 blocked TNF\(\alpha\)-induced cell death and improved the rate of cell survival up to 70% (Fig. 3B), which was comparable with the neutralizing antibody treatment. In contrast, A8 had no effect on TNF\(\alpha\)-induced cytotoxicity. We also found C87 had minimal effect on cell proliferation (data not shown). To ask whether C87 specifically blocks TNF\(\alpha\)-induced cytotoxicity, we assessed the effects of C87 on cell death induced by VP16 and ADR, both of which are chemotherapeutic drugs. We found that C87 exerted no detectable effects on ADR and VP16-caused cell death in L929 cells (Fig. 3C), HL60, and K562 cells (data not shown), even with doses up to 100 \(\mu\)M. Furthermore, the effect of C87 was also tested in Jurkat cells, in which a Fas antibody induced cell death in a dosage-dependent manner as
described previously (data not shown). C87 did not block the Fas antibody-caused cell death (Fig. 3D).

In addition, because of the high structural similarity between TNFα/H9251 and TNFα/H9252 and the ability to bind to the same receptor, we tested whether C87 was able to also block the function of TNFα/H9252 and found that C87 also blocked TNFα/H9252-induced cell death in L929 cells (Fig. 3E). This is not surprising given that the site of TNFR chosen for virtual screening can interact with both TNFα and TNFβ. Nevertheless, overexpression of TNFα, rather than TNFβ, was closely associated with a variety of diseases. Therefore, in the following experiments, we mainly focused on addressing the effect and the mechanism of action of C87 on TNFα-induced signaling.

**C87 Blocks Multiple TNFα-induced Signaling Pathways**—Having shown that C87 potently inhibits TNFα-induced cell death, we tested the effects of C87 on signaling transduction pathways triggered by TNFα. We first explored whether C87 could block the activation of caspase-3 and caspase-8, widely used readouts for TNFα signaling (23, 24). We found that C87 completely blocked TNFα-induced activation of caspase-3 and caspase-8 (Fig. 4, A and D). In contrast, A8, which was ineffective for TNFα-induced cell death, had no detectable effects. In addition, the activity of c-Jun N-terminal kinase (JNK), another key mediator of TNFα signaling (25–27), was significantly reduced by C87 in L929 cells (Fig. 4B). C87 also prevented the degradation of IκBα in cells treated with TNFα (Fig. 4C).

To further examine whether C87 was able to affect the expression of previously described downstream targets of TNFα signaling, we measured the mRNA levels of the KC gene (CXCL1), TNFα, IL-1α, and MIP-2 gene in L929 cells with or without C87 treatment. We found that C87 significantly suppressed the up-regulation of KC, TNFα, and IL-1α triggered by TNFα stimulation in L929 cells as early as 1 h after treatment (Fig. 5). This suppression was more consistent and robust 6 h after treatment (Fig. 5). Interestingly, although MIP-2 expression was sharply up-regulated by TNFα and suppressed by C87 after 1 and 2 h of stimulation, its expression decreased to a much lower level 6 h after TNFα stimulation, rendering the effect of C87 inhibition relatively indiscernible. Notably, in all of the analyses conducted, C87 exhibited similar potency to the TNFα-neutralizing antibody. Taken together, our results demonstrated that C87 potently blocks multiple signaling transduction pathways and downstream target gene activation triggered by TNFα.
C87 Directly Binds to hTNFα—SPR has been widely used to measure the binding between mobile analytes and immobilized biomolecules without the use of labels. The signals can be acquired qualitatively and quantitatively. It was previously reported that Biacore 3000 (GE Healthcare) could be used to provide kinetic data for the interactions between a drug and its target protein, prompting us to examine whether C87 directly binds to hTNFα (28). hTNFα was purified with its biological activity verified and subsequently used for SPR analysis. We found that the KD of C87 was 110 nM, nearly 100 times lower than the control (A8) (KD = 9 μM) (Fig. 6A). Thus, C87 binds directly to and has a high affinity for hTNFα. To further test whether the direct binding between C87 and TNFα was able to abolish the interaction between TNFα and its receptors, we took advantage of a TNFR1/2 competition ELISA assay. We found that an anti-TNFα antibody successfully abolished the interactions between TNFα and TNFR1 and TNFR2 (Fig. 6B, right panel), providing a validation for this assay. Surprisingly, we found that C87 failed to block TNFα binding to both TNFR1 and TNFR2 in the competitive assay (Fig. 6B, left panel). Thus, we speculated that instead of preventing TNFα-TNFR1/2 interaction, the direct binding of C87 with TNFα might disrupt the function of the TNFα-TNFR1/2 complex, thus leading to the strong inhibition of TNFα-induced signaling by C87.

C87 Attenuates Inflammation in Hepatitis Mouse—One of the most important criteria for a potential anti-TNFα drug is its efficacy in vivo. Thus, we took advantage of a hepatitis mouse model reported previously and explored the potential anti-inflammation effect of C87. Briefly, the TNFα inhibitor Enbrel or C87 was injected into BALB/c mice three times as described (Fig. 7A), and LPS/GalN were used subsequently to induce the onset of inflammation (Fig. 7A). Blood and liver tissues were collected for further analysis. Consistent with earlier reports, the level of plasma TNFα reached the peak in 1 h in mice after they were challenged with LPS/GalN (data not shown). We found that LPS/GalN injection caused rapid induction of liver inflammation and death of animals as early as 7 h after injection. The application of Enbrel led to survival of nearly all animals (95.8 ± 4.2%, Fig. 7B). Remarkably, C87 injection delayed the incidence of death and increased the survival rate by two folds, when compared with the vehicle control (58.5 ± 8.3% versus 20.8 ± 4.2%, Fig. 7B). By using histopathological analysis, we found treatment with C87 or Enbrel significantly reduced the liver damage and emergency of hemorrhage, whereas LPS/GalN treatment caused severe liver damage and wide hemorrhagic necrosis (Fig. 7, C and D). Furthermore, the level of alanine transaminase and aspartate transaminase was also consistently reduced in mice with C87 treatment (Fig. 7, E and F). Taken together, these results suggest that C87 is effective in attenuating TNFα-induced inflammation in vivo.

FIGURE 6. The mechanism of the hTNFα-antagonistic activity of C87. A, SPR binding kinetic curve was determined with different concentration of C87 and control compound (A8). hTNFα was coupled to HRBio chips, and C87 was applied from 2 to 16 μM. RU, resonance unit. B, C87 failed to inhibit the binding of hTNFα and hTNFR1/2 in vitro. An ELISA was used to measure inhibition of solution-phase hTNFR1/2-His tag binding to hTNFα immobilized on a microtiter plate by serial dilutions of C87 and an anti-TNFα antibody as the positive control.
DISCUSSION

Target identification, screening, and optimization of lead candidates are essential early steps in drug discovery. In this study, we applied the computer-aided drug design technique to the screening of small-molecule antagonists against TNFα/H9251, enabling us to identify a lead compound (C87) that potently inhibits TNFα/H9251-induced cytotoxicity. More importantly, the compound effectively attenuates TNFα/H9251-induced inflammation in vivo and significantly improves survival of animals. This is one of the first effective small-molecule inhibitors of TNFα/H9251 identified to date and can be potentially used for therapeutic interventions of TNFα activities for clinical purposes. Our study also highlights the effectiveness of combining virtual screening with functional assays for the discovery and development of novel small-molecule drugs that target TNFα signaling and potentially other signaling pathways implicated in human diseases.

The three-dimensional structures of TNFα and TNFβ, which share the same receptors, are strikingly similar. Therefore, in this study, we took advantage of the crystal structure of TNFβ-TNFRI complex and utilized it as the computer-aided drug design basis for screening. The key sites of TNF-TNFRI interaction include three essentials loops: loop 1 of domain 2 (Phe60-His66), loop 2 of domain 2 (Cys76-Val83), and loop 1 of domain 3 (Trp107-Leu111) (16, 29). C87 mimics the loop structure of TNFR1 from Arg77 to Val83: one of the key TNF-binding sites. Takasaki et al. (19) previously used a similar strategy and identified an exocyclic peptide referred to as WP9Y, which mimics the loop of TNFR1 from Tyr107 to Leu111, forms a cyclic structure via a disulfide bond and inhibits TNFα cytotoxicity by binding to its receptors. Our study extended this earlier work and successfully identified a small-molecule TNFα inhibitor, C87, which specially blocks the signal transduction of TNFα-TNFRI via directly binding to TNFα (30–32).
In virtual screening, computational modeling theoretically suggests that C87 mimics the structure of the loop 2 within domain 2 of TNFR1, which interacts with TNFα. Subsequently, we confirmed that C87 can bind to TNFα with high affinity using the SPR assay (33–35), and it is capable of inhibiting the signaling transduction, downstream gene expression, and cytotoxicity of TNFα in vitro and in vivo. But surprisingly, we found that C87 fails to effectively block TNFα binding to TNFR1/2 in experiments with the ELISA assay. Unlike the functional mechanism of C87, Carter et al. (36) utilized a TNFα/TNFR1-binding assay to conduct library screening and identified one chemical compound named as IW927, which shows 2000-fold higher selectivity for TNFR1 over TNFR2 and functions through covalent modification of TNFR1 to disrupt TNFα/TNFR1 interaction (36, 37). In addition to disrupting TNFα/TNFR interaction, perturbation of TNFα trimerization has been an alternative strategy to developing TNFα inhibitors. For example, He et al. (38) synthesized a compound named as SPD305, which inhibits TNFα/TNFR1 interaction and blocks TNFα-mediated IkBα degradation in HeLa cells. X-ray crystallography revealed that SPD305 displaces a subunit from the TNFα trimer (38). Thus, based on the results above, we could not exclude the possibility of disrupting trimerization of TNFα by C87. Alternatively, C87 could directly bind to TNFα and might be sandwiched between TNFα and TNFR, and its binding might lead to conformational changes that perturb the function of the TNFα-TNFR complex and therefore impair the signaling activities downstream of TNFα. Further experimentation with x-ray crystallography should reveal structural insights into how C87 might interact with TNFα and TNF receptors and thus may provide mechanistic explanations for the function of C87.

Importantly, in addition to its efficacy in vitro, C87 significantly attenuates TNFα-induced inflammation in vivo and thus holds promise as a potential therapeutic reagent for TNFα-mediated inflammatory diseases. We took advantage of the LPS and GalN-induced acute liver injury mouse model to determine the in vivo function of C87. In this model, combined injection of GalN and LPS leads to lethality of mice. TNFα plays a key role in this lethality, which can be almost completely protected by the anti-TNFα neutralizing antibody (39, 40). We found that similar to C87, another previously described TNF chemical inhibitor, SPD305 (6,7-dimethyl-3-[(methyl-(2-{methyl-[1-(3-trifluoromethyl-phenyl)-1H-indol-3-ylmethyl]-amino-ethyl]-amino)-methyl]-chromen-4-one), also attenuated inflammation in the hepatitis mouse model (data not shown). The survival rate was slightly higher than C87 (58.3 ± 8.3% versus 62.5 ± 12.5%). However, the anti-inflammatory effects of both C87 and SPD305 were lower than Enbrel. The relatively weaker effect of C87 may result from its low solubility and/or instability in vivo. Structural optimization of C87 to improve its properties and efficacy awaits future experimentation.

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