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Discovery of the inhibitors of tumor necrosis factor alpha with structure-based virtual screening

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ABSTRACT

Tumor necrosis factor alpha (TNF-α) has been considered as one of the attractive drug targets for allergic diseases including asthma. We have been able to identify five novel TNF-α inhibitors with a drug-design protocol involving the structure-based virtual screening and in vitro cell-based assay for antagonistic activity. Because the newly discovered inhibitors are structurally diverse and have the desirable physicochemical properties as a drug candidate, they deserve a further investigation as anti-asthmatic drugs. The interactions of the identified inhibitors in the binding site of TNF-α dimer are addressed in detail to understand the mechanisms for the stabilization of the inactive dimeric form of TNF-α.

Most of the patients suffering from the severe asthma need an emergency care for the upper airway dyspnea that results from the unexpected bronchoconstriction and hyper-responsiveness.[1] A variety of cytokines are known to provoke the severe asthma. Among them, tumor necrosis factor alpha (TNF-α) plays a key role in the recruitment of inflammatory leukocytes that leads to the airway remodeling involving the destruction of tissue.[2–4] It participates in the inflammatory process as a multifunctional cytokine promoting the binding of the circulating leukocytes to the endothelial cells of the airway. This process has an effect of increasing the thickness and stiffness of airway tissue in an irreversible fashion, which culminates in the severe and chronic asthma.[5,6]

The biopsies of the severe asthma patients have been known to contain a high mRNA expression level of TNF-α,[7] which indicates that it can be a promising target for the discovery of anti-asthmatic drugs. In this regard, some monoclonal antibodies and a circulating receptor fusion protein were developed for the clinical treatment of asthma using TNF-α as the target protein. These protein drugs have also been effective in the treatment of proinflammatory disease including rheumatoid arthritis and Crohn's disease,[8] further motivating the development of the new inhibitors of TNF-α.

A few years ago, He et al. reported the X-ray crystal structure of TNF-α in complex with a small-molecule inhibitor.[9] It was shown that the inhibitor would promote the formation of the inactive dimeric form of TNF-α by displacing a subunit of the trimer in the active form. A useful method of in vitro cell-based assay for TNF-α activity was also reported, which has made it possible to identify the novel TNF-α inhibitors with chromen and tyrosine-proline based peptidomimetic scaffolds.[10,11] Nonetheless, the discovery of TNF-α inhibitors has lagged behind the pharmacological and structural studies. Only a few additional structural classes of TNF-α inhibitors have been reported so far. Several derivatives of methyl jasmonate and *N*-2,4-pyridine-*N*-phenyl-*N*′-alkyl urea scaffolds were identified as TNF-α inhibitors, some of which proved to have the nanomolar inhibitory activities.[12–14] Recently, a group of phosphodiesterase-4 inhibitors including phthalimide and sulfone groups have also been shown to be the potent inhibitor of TNF-α, which indicates that they can be a promising drug candidate for the treatment of asthma.[15]

In the present study, we identified the novel classes of TNF-α inhibitors by means of a structure-based drug-design protocol involving the virtual screening with docking simulations and in vitro cell-based immunoassay. Because TNF-α is active and binds to TNF-α receptor in a trimeric form, its putative inhibitors can be selected in order to prevent the formation of trimer by stabilizing the inactive dimeric form. In this regard, the compounds that bind tightly at the interface of TNF-α dimer can be good candidates for the TNF-α inhibitors. The characteristic feature that discriminates our virtual screening approach from the others lies in the implementation of an accurate solvation model in calculating the binding free energy between TNF-α and its putative ligands, which would have an effect of increasing the accuracy in virtual

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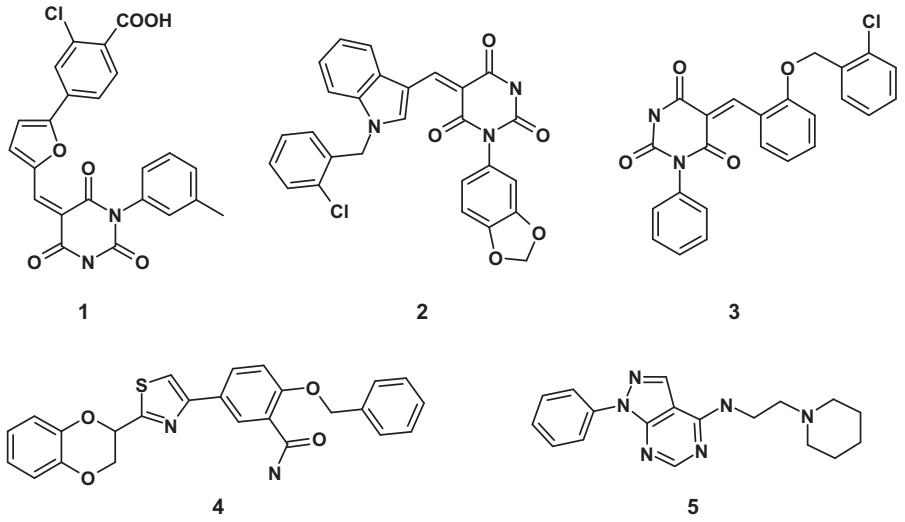

Figure 1. Chemical structures of the newly identified TNF-α inhibitors.

screening.[16] It was shown that the docking simulation with the improved binding free energy function could be a useful tool for elucidating the activities of the identified inhibitors, as well as for enriching the chemical library with molecules that are likely to have desired biological activities.

Three-dimensional co-ordinates in the X-ray crystal structure of TNF-α dimer in complex with an inhibitor (PDB code: 2AZ5) were selected as the receptor model in the virtual screening with docking simulations.[17] The docking library for TNF-α comprising about 240,000 compounds was constructed from the latest version of the chemical database distributed by Interbioscreen (http://www.ibscreen.com) containing approximately 460,000 synthetic and natural compounds. Prior to the virtual screening with docking simulations, they were filtrated on the basis of Lipinski's 'Rule of Five' with the ISIS/BASE program of version 2.4 to adopt only the compounds with the physicochemical properties of potential drug candidates[18] and without reactive functional group(s). All of the compounds included in the docking library were then subjected to the CORINA program to generate their 3-D atomic co-ordinates,[19] followed by the assignment of Gasteiger–Marsilli atomic charges.[20] We used the AUTODOCK program[21] in the virtual screening of TNF-α inhibitors because the outperformance of its scoring function over those of the others had been shown in several target proteins.[22] AMBER force field parameters were assigned for calculating the van der Waals interactions and the internal energy of a ligand as implemented in the AUTODOCK program. Docking simulations were then carried out in the binding site located at the interface of TNF-α dimer to score and rank the compounds in the docking library according to their calculated binding affinities for the dimer. In the actual docking simulations, we used the empirical AUTODOCK scoring function improved by the implementation of a new solvation model for a molecule. The modified scoring function has the following form:[23]

$$\Delta G^{\mathrm{aq}}_{\mathrm{bind}} = W_{\mathrm{vdW}} \sum_{i=1} \sum_{j>i} \left(\frac{A_{ij}}{r_{ij}^{12}} - \frac{B_{ij}}{r_{ij}^{6}} \right) + W_{\mathrm{hbond}} \sum_{i=1} \sum_{j>i} E(t) \left(\frac{C_{ij}}{r_{ij}^{12}} - \frac{D_{ij}}{r_{ij}^{10}} \right) + W_{\mathrm{elec}} \sum_{i=1} \sum_{j>i} \frac{q_i q_j}{\varepsilon(r_{ij}) r_{ij}} + W_{\mathrm{tor}} N_{\mathrm{tor}} + W_{\mathrm{sol}} \sum_{i=1} S_i \left(\mathrm{Occ}_i^{\max} - \sum_{j>i} V_j e^{-\frac{r_{ij}^2}{2\sigma^2}} \right) \quad (1)$$

Of the 240,000 compounds subject to the virtual screening with docking simulations, 100 top-scored compounds were selected as the virtual hits. Eighty one of them were available from the compound supplier and were tested for the inhibitory activity against TNF-α.[27] Among the screened compounds, five revealed more than 45% inhibition at the concentration of 10 μM. The chemical structures and the inhibitory activities of these newly identified TNF-α inhibitors are shown in Figure 1 and Table 1, respectively. Also, the physicochemical properties of these new TNF-α inhibitors are listed in Table 2 to provide insight into the estimation of their drug-likenesses. It is noted that none of these newly found inhibitors are structurally analogous to the known inhibitors. Compounds **1–3** contain the pyrimidine-2,4,6-trione moiety in common, which indicates that it can be a promising pharmacophore for TNF-α inhibitors. The in vitro antagonistic activities range from 45% to 65% in LPS-activated Raw264.7 macrophage cells. To the best of our knowledge, these compounds have not been reported as TNF-α inhibitors so far neither in the literature and nor in the patents. Furthermore, they are structurally diverse as well as have desirable physicochemical properties as a drug candidate, and therefore each of the five inhibitors can be considered as a new inhibitor scaffold for further development by a structure–activity relationship or de novo design methods.

To estimate the accuracy of the scoring function of the modified AUTODOCK program in predicting the binding modes of the inhibitors at the interface of TNF-α dimer, we examined the reproducibility of the bound conformation in the original X-ray crystal structure (PDB ID: 2AZ5)[9] for the known inhibitor. The root mean square deviation from the bound conformation in the X-ray structure falls within 1.5 Å for all of the binding conformations in the lowest-energy cluster. This validation result indicates that the binding mode of a TNF-α inhibitor at the interface of the dimer can be estimated from the docking simulation with the scoring function in Eq. 1.

To obtain some energetic and structural insight into the inhibitory mechanism of the newly identified TNF-α inhibitors, their binding modes in the ligand-binding site of TNF-α dimer were investigated using the AUTODOCK program with the procedure described above. Figure 2 shows the best-scored AUTODOCK conforma-

Table 1
Inhibitory activities of the newly identified inhibitors **1**–**5** and the reference against TNF-α

Compound	% inhibition at 10 μM
Baicalein	71.3 ± 4.1
1	64.0 ± 2.3
2	54.6 ± 5.7
3	47.9 ± 3.6
4	52.6 ± 3.9
5	45.8 ± 0.8

Table 2
Physicochemical properties of the newly identified TNF-α inhibitors

Compound	Molecular weight	Number of hydrogen bond donors	Number of hydrogen bond acceptors	Number of rotatable bonds	*C* log *P*
1	450.8	2	8	4	3.77
2	499.9	1	8	4	4.70
3	444.5	1	6	6	4.74
4	432.9	1	6	5	4.45
5	322.4	1	6	5	3.59

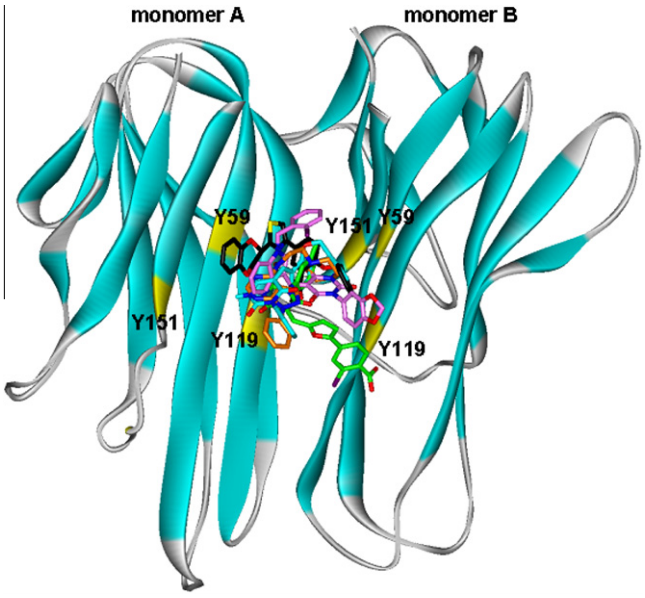

Figure 2. Comparative view of the binding modes of **1**–**5** in the binding site of TNF-α dimer. Carbon atoms of **1**, **2**, **3**, **4**, and **5** are indicated in green, pink, cyan, black, and orange, respectively. Indicated in yellow are the positions of the three tyrosine residues that are known to play a key role in ligand binding. This figure was prepared with the VIEWERPRO program.

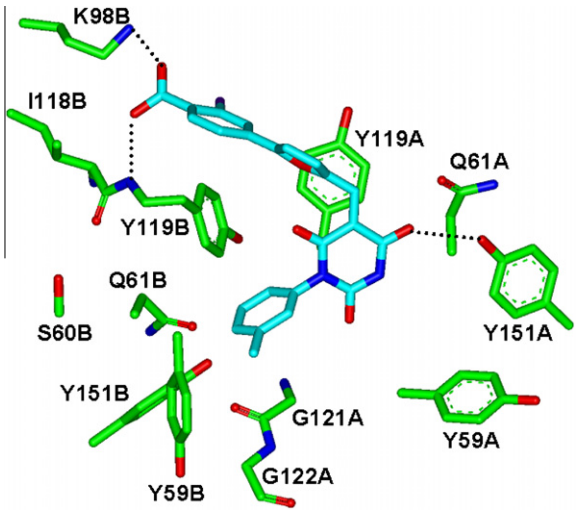

Figure 3. Calculated binding mode of **1** in the binding site at the interface of TNF-α dimer. Carbon atoms of the protein and the ligand are indicated in green and cyan, respectively. Each dotted line indicates a hydrogen bond. This figure was prepared with the VIEWERPRO program.

tions of **1–5** in the gorge of ligand-binding site residing at the interface of TNF-α dimer. As revealed by the superposition of the docked structures, all of the inhibitors seem to be well-accommodated in the binding site involving the three tyrosine residues (Tyr59, Tyr119, and Tyr151) that have been shown to play a critical role in ligand binding.[9] In order to examine the possibility of the allosteric inhibition of TNF-α by the inhibitors, docking simulations were carried out with the grid maps for the receptor model so as to include the entire part of TNF-α dimer. However, the binding configuration in which an inhibitor resides outside the binding site was not observed for any of the five inhibitors. This result supports the possibility that the inhibitors would impair the activity of TNF-α through the stabilization of its inactive dimeric form.

The calculated binding mode of the most potent inhibitor **1** in the binding site of TNF-α dimer is shown in Figure 3. To be consistent with the criterion for choosing the hit compounds in virtual screening, we selected the best-scored pose of **1** as the most probable binding mode. In this calculated TNF-α dimer-**1** complex, the two oxygen atoms of the terminal carboxylate group of the inhibitor appear to form the hydrogen bonds at the interface of the dimer with the side-chain ammonium ion moiety of Lys98 (K98B) and the backbone amidic group of Tyr119 (Y119B) in the monomer B. The presence of these multiple hydrogen bonds indicates that the carboxylate group can play a role of anchor for binding of **1** to the TNF-α dimer. A stable hydrogen bond is also established between one of the aminocarbonyl oxygens of pyrimidine-2,4,6-trione moiety of **1** and the side-chain phenolic oxygen of Tyr151 in the monomer A (Y151A). The inhibitor **1** may be further stabilized in binding site through the hydrophobic interactions with the nonpolar residues including the three tyrosine residues for ligand binding in both motifs and the side chain of an isoleucine residue in the monomer B (I118B). Thus, the simultaneous establishment of the multiple hydrogen bonds and the hydrophobic interactions in the binding site can be invoked to explain the highest inhibitory activity of **1** against TNF-α.

Figure 4 shows the most stable binding mode of **4** in the binding site of the TNF-α dimer. The binding mode of **4** differs from that of **1** in that the side chain of Lys98B is not involved in the hydrogen bond with the inhibitor. In this calculated TNF-α dimer-**4** complex, the oxygen atom and the amide group attached to the central phenyl ring receives and donates a hydrogen bond from the side chain of Tyr151B and to the backbone aminocarbonyl oxygen of Tyr119A, respectively. The importance of the capability to form the hydrogen bonds with the Tyr residues at the interface of dimer has been well appreciated in the previous X-ray crystallographic studies for the stabilization of the inactive dimer.[9] As in the TNF-α dimer-**1** complex, the side-chain phenolic group of Tyr151A serves as a hydrogen bond donor with respect to an oxygen atom of the inhibitor. It is also a common structural feature of TNF-α dimer-**1** and TNF-α dimer-**4** complexes that the inhibitor is stabilized by hydrophobic interactions with the three tyrosine residues and the other nonpolar residue residing around the binding site (Leu57A in the latter case). Although the number of the hydrogen bonds appears to be the same in TNF-α dimer-**1** and TNF-α dimer-**4** complexes, they seem to be established in a stronger form in the former than in the latter due to the involvement of ionic groups. Thus, the weakening of the hydrogen-bond interactions should be responsible for the lower inhibitory activity of **4** than **1**.

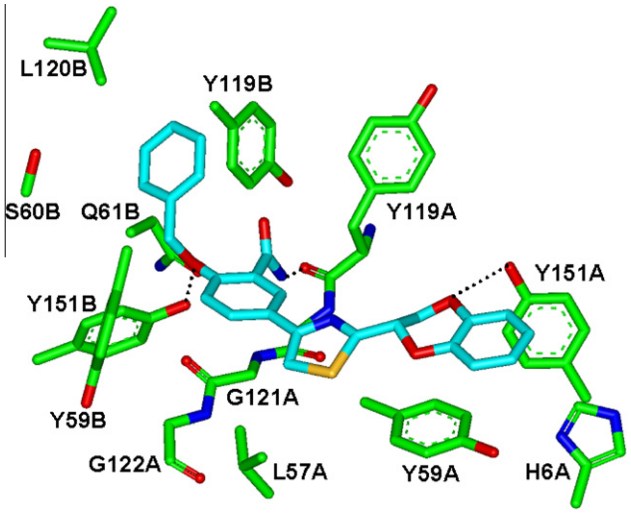

Figure 4. Calculated binding mode of **4** in the binding site at the interface of TNF-α dimer. Carbon atoms of the protein and the ligand are indicated in green and cyan, respectively. Each dotted line indicates a hydrogen bond. This figure was prepared with the VIEWERPRO program.

In conclusion, we have identified five novel inhibitors of TNF-α by applying a computer-aided drug-design protocol involving the structure-based virtual screening with docking simulations under consideration of the effects of ligand solvation in the scoring function. We thus report the first example for the successful application of the structure-based virtual screening to identify the TNF-α inhibitors. The newly found inhibitors are structurally diverse and have desirable physicochemical properties as a drug candidate. Therefore, they seem to deserve consideration for further development to discover new anti-asthmatic drugs. Detailed binding mode analyses with docking simulation show that the inhibitors can stabilize the inactive TNF-α dimer in the binding site at the interface through the formation of multiple hydrogen bonds and the establishment of hydrophobic contacts in a simultaneous fashion.

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17. After removing the ligand and solvent molecules from the original X-ray crystal structure, hydrogen atoms were added to each protein atom using the AMBER program of version 7. A special attention was paid to assign the protonation states of the ionizable Asp, Glu, His, and Lys residues in the X-ray structure of TNF-α dimer. The side chains of Asp and Glu residues were assumed to be neutral if one of their carboxylate oxygens pointed toward a hydrogen-bond accepting group including the backbone aminocarbonyl oxygen at a distance within 3.5 Å, a generally accepted distance limit for a hydrogen bond of moderate strength. Similarly, the lysine side chains were assumed to be protonated unless the terminal amine group was in proximity of a hydrogen-bond donating group. The same procedure was also applied to determine the protonation states of the two nitrogen atoms on the imidazole ring of His residues.
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23. Here, W_{vdW}, W_{hbond}, W_{elec}, W_{tor}, and W_{sol} are the weighting factors of van der Waals, hydrogen bond, electrostatic interactions, torsional term, and desolvation energy of the putative inhibitors, respectively. r_{ij} represents the interatomic distance, and A_{ij}, B_{ij}, C_{ij}, and D_{ij} are related to the depths of the potential energy well and the equilibrium separations between the protein and ligand atoms. The hydrogen bond term has an additional weighting factor, $E(t)$, representing the angle-dependent directionality. Cubic equation approach was applied to obtain the dielectric constant required in computing the interatomic electrostatic interactions between TNF-α and a ligand molecule.[24] In the entropic term, N_{tor} is the number of sp^3 bonds in the ligand. In the desolvation term, S_i and V_i are the solvation parameter and the fragmental volume of atom i,[25] respectively, while Occ_i^{max} stands for the maximum atomic occupancy. In the calculation of molecular solvation free energy term in Eq. 1, we used the atomic parameters developed by Kang et al.[26] because those of the atoms other than carbon were unavailable in the current version of AUTODOCK. This new solvation model proved to be useful for a rapid calculation of the molecular solvation free energies with accuracy; the squared correlation coefficients with respect to the experimental data were larger than 0.85 for both training and test sets.[26] The modification of the solvation free energy term is expected to increase the accuracy in virtual screening because the underestimation of ligand solvation often leads to the overestimation of the binding affinity of a ligand with many polar atoms.
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27. With respect to in vitro cell-based assay for antagonistic activity of the putative TNF-α inhibitors, we followed the procedure used by Jung and co-workers.[12] At first, all of 81 compounds selected from the precedent virtual screening were diluted in dimethylsulfoxide solvent to prepare the solution with the final concentration of 10 μM at which the cell-based immunoassays were carried out. Raw264.7 cells, a mouse murine macrophage cell line purchased from Korean Cell Line Research Foundation (KCLRF), were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were grown to 80–90% confluence in this study, and were incubated at 37 °C. Raw264.7 cells were seeded in the 6-well plates as dense as 5×10^5 cells/well and incubated in a humidified atmosphere containing 5% CO_2. The cells were then exposed to lipopolysaccharide (LPS) solution (1 μg/mL) to activate TNF-α receptors and thereby to produce the cell types for allergic asthma. After 14 h from the addition of LPS, the compounds selected from the virtual screening were added to each well and incubated for 2 h to check their inhibitory activities against TNF-α. After staining of the cells with anti-CD 120a antibody and PE-conjugated streptavidin for 1.5 h at 4 °C in the dark, the inhibition of the activity of TNF-α was analyzed with fluorescence activated cell sorting (FACS) techniques. Such a FACS analysis can be useful in this study because the higher is the activity of TNF-α in the cells, the higher should be the fluorescence intensity of the cells. Mean fluorescence intensity of the cells was thus determined on FACScan with the Cell Quest program to estimate the inhibitory activities of the selected compounds. One-way analysis of variance was used to test the statistical significance between groups. *P*-Values lower than 0.05 were considered statistically significant. The SPSS program of version 13.0 was used for these statistical analyzes. In this in vitro immunoassay, the known TNF-α inhibitor baicalein was used as the reference.