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Structure-based discovery of a novel non-peptidic small molecular inhibitor of caspase-3

Junichi Sakai,[a] Atsushi Yoshimori,[b] Yasuyo Nose,[a] Akihiko Mizoroki,[a] Naoyuki Okita,[c] Ryoko Takasawa[a] and Sei-ichi Tanuma[a,c,*]

[a]*Department of Biochemistry, Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan*
[b]*Institute for Theoretical Medicine, Inc., Tokyo Institute of Technology, Yokohama Venture Plaza W101, 4259-3 Nagatsuda, Midori-ku, Yokohama, Kanagawa 226-8510, Japan*
[c]*Genome and Drug Research Center, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan*

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Abstract—Ac-DNLD-CHO is a novel caspase-3 specific peptide inhibitor that was rationally designed by our computational strategy. The specificity was shown to be due to the specific interaction of NLD moiety with the active site of caspase-3 on the basis of docking mode and site-directed mutagenesis analyses. Here, we computationally screened non-peptidic small molecular inhibitors of caspase-3 from our chemical library using a reliable pharmacophore derived from the specific binding mode of NLD. Through in vitro enzyme assay of the screened candidate compounds, we discovered a novel caspase-3 specific small molecular inhibitor, CS4566, which has a unique scaffold structure. The binding mode of CS4566 to caspase-3 mimics that of NLD, especially LD moiety. This represents a promising lead compound for creating non-peptidic pharmaceuticals for caspase-mediated diseases, such as neurodegenerative disorders.

1. Introduction

Apoptosis is a major process of cell death that is important for development and defense in eukaryotic organisms.[1–3] Its process is characterized by a series of apoptotic morphological alterations and nucleosomal DNA fragmentation, which is catalyzed by a family of cysteine aspartyl proteases termed caspases. Inappropriate control of the apoptosis machinery has been implicated in many diseases, including cancer, autoimmune diseases, and neurodegenerative disorders.[4,5] Therefore, key apoptosis factors are attractive molecular targets for designing specific pharmaceuticals for the apoptosis diseases.

Caspases are normally expressed as zymogens, and their activations are caused by their specific proteolytic cleavages.[6–13] In pathological conditions, such as ischemic brain injury and amyotrophic lateral sclerosis (ALS), main apoptotic caspases, particularly caspase-3, are activated and cause excessive neuronal cell death.[4,5,14–20] Hence, the inhibitors of caspases have been expected to be beneficial pharmaceuticals for neurodegeneration. Although many peptide and peptide mimetic inhibitors of caspases have been reported,[21–25] they have intrinsic limitations for therapeutic use because of their poor selectivity, cell-permeability, in vivo stability, and bioavailability. Here, we describe the computational development of non-peptidic small molecular inhibitors of caspase-3 by peptide mimetics using caspase-3 specific peptide inhibitors.

Previously, we developed a new computational program for designing of optimized binding peptides to the target domains (hot spots) on target proteins, named amino acid positional fitness (APF) method, and designed a novel casapse-3 specific peptide inhibitor, Ac-DNLD-CHO.[26,27] So, we attempted computationally to design small molecular inhibitors of caspase-3 based on the binding mode of DNLD peptide. Searching for DNLD peptide mimetics by structure-based virtual screening (SBVS), we discovered a novel caspase-3 non-peptidic

Keywords: Caspase-3; Apoptosis; Inhibitor; DNLD; Structure-based virtual screening (SBVS); Conversion to small molecules through optimized-peptides strategy (COSMOS).
* Corresponding author. Tel.: +81 4 7124 1501; fax: +81 4 7121 3620; e-mail: tanuma@rs.noda.tus.ac.jp

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small molecular inhibitor, 4-(ethoxycarbonylmethoxy)-1-hydroxy-2-naphthoic acid, named CS4566, which has a unique scaffold structure. This novel class of small molecular inhibitor of caspase-3 is a promising lead compound for creating non-peptidic small molecular inhibitors and pharmaceuticals for caspase-mediated diseases.

2. Results

2.1. Structural definition of caspase-3 pharmacophore based on the predicted binding mode of Ac-NLD-CHO with caspase-3

In our previous study, we designed a novel caspase-3 specific peptide inhibitor, Ac-DNLD-CHO, using our computational molecular design technique.[26,27] It inhibited caspase-3 potently and specifically in vitro. Furthermore, caspase fluorogenic substrate, Ac-DNLD-MCA, was specifically cleaved by caspase-3, but not caspases-7, -8, and -9. These results suggest that DNLD is the *optimized binding peptide* to the active site of caspase-3, and expected to be a potential basis for the development of non-peptidic inhibitors of caspase-3. Furthermore, to understand the specificity and potency of Ac-DNLD-CHO for caspase-3, we analyzed its binding mode through a computational docking simulation.[27] In the active site of caspase-3, Asn in Ac-DNLD-CHO specifically interacts with Ser209 in the S_3 subsite, and Leu tightly interacts with the hydrophobic S_2 subsite. These data suggest that the specificity and potency of DNLD peptide is caused by the specific interactions of NL moiety with the active site of caspase-3. Therefore, we decided to use Ac-NLD-CHO as a molecular basis for design of non-peptidic small molecular inhibitors of caspase-3.

In order to design scaffolds of NLD mimic small molecules, we first defined the pharmacophore of NLD based on the binding mode of Ac-NLD-CHO with caspase-3. In the S_1 subsite of caspase-3, Asp in NLD forms three hydrogen bonds with Arg64 and Gln161 (Fig. 1). Moreover, Leu in NLD is tightly bound to the S_2 subsite based on hydrophobic interactions, and Asn forms a hydrogen bond with Ser209 in the S_3 subsite, which exists only in caspase-3 among all human caspases (Fig. 1).[27] From these characteristic interactions, the pharmacophore of NLD was defined (Fig. 2). Thus, we decided to use the pharmacophore as a filter for screening of mimetics from our chemical library.

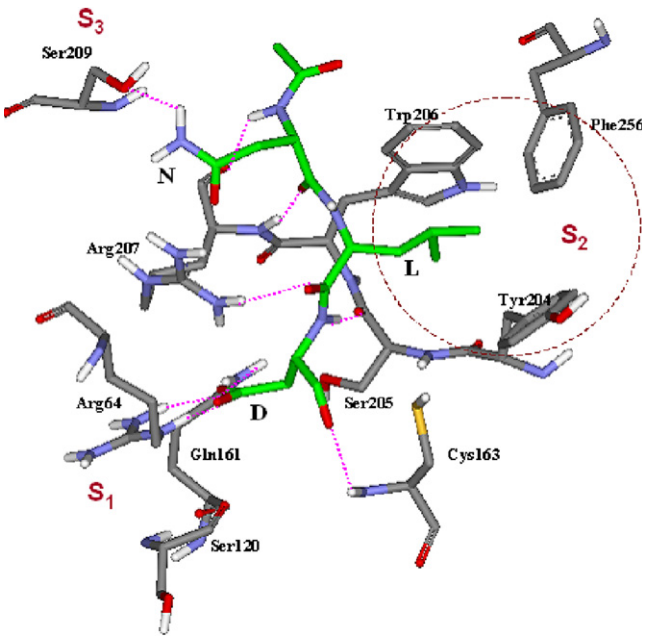

Figure 1. Predicted binding mode of Ac-NLD-CHO to caspase-3 active site. The binding mode was obtained from docking simulation. Nitrogen, oxygen, and carbon atoms of the inhibitors are illustrated in blue, red, and green, respectively. Hydrogen bonds are shown as pink dashed lines. Hydrophobic interaction is shown as a red dashed circle. S_1, S_2, and S_3 show binding pockets of caspase-3 active site.

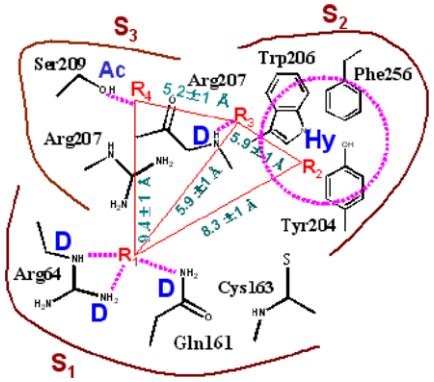

Figure 2. Defined pharmacophore of Ac-NLD-CHO. R_1, R_2, R_3, and R_4 show the positions where the important regions of ligands are placed, and the numerals show distances between them. Hydrogen bonds are shown as pink dashed lines. Donors and acceptors of the hydrogen bonds are shown as 'D' and 'Ac', respectively. The hydrophobic region of caspase-3 active site is shown as 'Hy', and hydrophobic interactions are shown as a pink dashed circle.

2.2. Computational screening of NLD mimetic inhibitors of caspase-3

Based on the pharmacophore of NLD, we performed SBVS in our comprehensive chemical library containing about 500,000 commercially available compounds. From this library, the 400,000 compounds were selected by drug likeness analysis based on 'Lipinski's rule of five'. Then, the selected compounds were filtered through the NLD pharmacophore filter. Finally, 50 compounds, which had the high docking energy scores and could mimic the binding mode of NLD to caspase-3 active site, were subjected to enzyme inhibition assays.

Twenty-eight out of the 50 showed inhibitory activity against caspase-3 with IC_{50} values ranging from 10 to 200 μM (data not shown). Of these compounds, we focused on one promising compound, named CS4566 (Fig. 3A). This compound inhibited caspase-3 with IC_{50} value of 13.6 μM (Fig. 3C, Table 1). In contrast, it had little inhibitory effect on caspases-7, -8, and -9

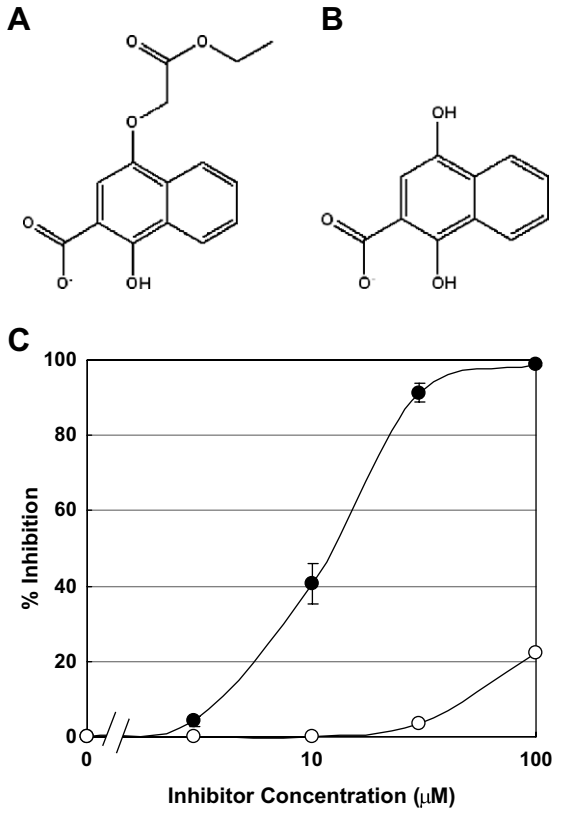

Figure 3. Evaluation of a new caspase-3 inhibitor, CS4566, through in vitro enzymatic assay. (A) Chemical structure of CS4566. CS4566; 4-(ethoxycarbonylmethoxy)-1-hydroxy-2-naphthoic acid. (B) Chemical structure of 1,4-dihydroxy-2-naphthoic acid. (C) Caspase-3 inhibitory activities of CS4566 and 1,4-dihydroxy-2-naphthoic acid. Recombinant human caspase-3 was preincubated for 10 min with indicated concentrations of CS4566 (●) and 1,4-dihydroxy-2-naphthoic acid (○), and then the activities of the caspase-3 were measured as described in Section 4. Values are averages of four independent experiments and shown with standard deviations (SD).

(Fig. 4, Table 1). It has a unique scaffold structure and its binding mode to caspase-3 mimics the binding mode of NLD, especially LD moiety very well (Fig. 5). As a control experiment, 1,4-dihydroxy-2-naphthoic acid (Fig. 3B), which does not have ester moiety possessed in CS4566, was subjected to enzymatic assay. This compound poorly inhibited caspase-3 (Fig. 3C), suggesting that the binding mode of CS4566 with the active site of caspase-3 is adequate, and that the hydrophobic interaction between S_2 subsite of caspase-3 and the ester group of CS4566 is important for caspase-3 inhibitory ability of CS4566. These results suggest that CS4566 is the first non-peptidic small molecular inhibitor that is computationally designed from the data of the binding mode of the optimized caspase-3 specific peptide, DNLD.

3. Discussion

Our new concept for drug design is 'the conversion of the *optimized binding peptide* to non-peptidic small molecules by SBVS, and then the optimization of the small molecules by SBDD (structure-based drug design)' (named COSMOS, *co*nversion to *s*mall *m*olecules through *o*ptimized-peptides *s*trategy). In this strategy, the most optimized binding peptide is first computationally designed on a hot spot in the target protein. Second, the optimized binding peptide can be converted to small molecules by SBVS based on its pharmacophore. Third, the selected candidates are easily evaluated by in vitro assays. Finally, the discovered hit compounds are subjected to SBDD for optimization. It is anticipated that this strategy allows us to search for effective lead compounds for further drug design and optimization in lower cost and shorter time than in vitro large-scale screening methods, such as high-throughput screening (HTS), which are now generally performed.

In this study, we tried to convert the optimized caspase-3 specific peptide inhibitor, Ac-DNLD-CHO, to non-peptidic small molecular inhibitors, according to our concept. In the S_1 subsite of caspase-3, Asp (P_1 position) in DNLD forms three hydrogen bonds (Fig. 1). It is known that Asp in this position is essential for recognition of caspase substrates or peptide inhibitors.[29,30] Therefore, it is very important to identify non-peptidic structures which are able to mimic the interactions of Asp (P_1) with caspase-3 active site. Furthermore, our previous study showed that the interactions between NL residues in DNLD and caspase-3 active site are crucial for the potency and specificity of DNLD.[27] Thus, we decided to use the binding mode of Ac-NLD-CHO as a basis for the construction of a pharmacophore of caspase-3 specific non-peptidic inhibitors. When we selected key interactions, several hydrogen bonds in NLD back bone were omitted because the variation of molecular structures is limited by peptide linkages. Since it is very difficult to convert peptide back bones to other scaffolds to form hydrogen bonds, we put importance on the interactions of the peptide side chains rather than the main chain. According to this idea, we defined the pharmacophore of NLD as shown in Figure 2.

Using this pharmacophore as a filter, we performed virtual screening in our comprehensive chemical library. Fifty compounds were selected and then subjected to enzyme inhibition assay. Among them, 28 compounds showed inhibitory effect against caspase-3. CS4566, one focused compound, inhibited caspase-3 with IC_{50} value of 13.6 μM, suggesting that it is possible to identify inhibitors with IC_{50} values ranging at least 10–20 μM by using our pharmacophore. Moreover, it has little inhibitory activity on caspases-7, -8, and -9, suggesting the potential of the pharmacophore to discover selective inhibitors against caspase-3. These observations suggest that our defined pharmacophore is reliable, and that it is very useful for identifying small molecular inhibitors of caspase-3.

From the docking simulation between CS4566 and caspase-3, they formed five hydrogen bonds and tightly interacted by hydrophobic interactions (Fig. 5A). These interactions closely resemble those of NLD, especially Ac-LD-CHO (Fig. 5). It is unlikely that there is any compound in our chemical library which completely

Table 1. Effects of CS4566 on caspases-3, -7, -8, and -9

IC_{50} (μM)			
Caspace-3	Caspace-7	Caspace-8	Caspace-9
13.6 ± 1.99	84.9 ± 9.83	>100	93.0 ± 1.22

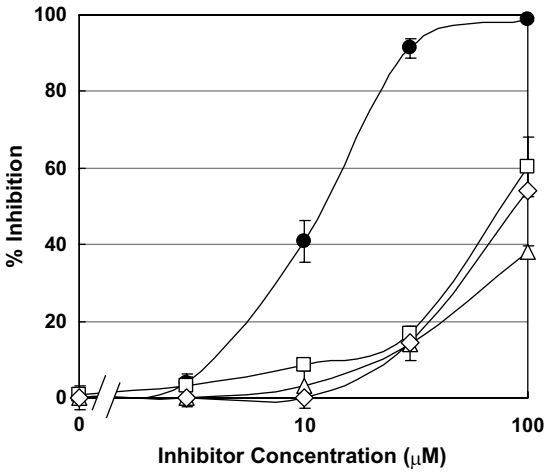

Figure 4. Inhibitory effects of CS4566 on caspases-3, -7, -8, and -9. Recombinant human caspase-3 (●), caspase-7 (□), caspase-8 (△), and caspase-9 (◇) were preincubated for 10 min with indicated concentrations of CS4566, and then the activities of the caspases were measured with each substrate as described in Section 4. Values are averages of four independent experiments and shown with SD.

corresponds to our defined pharmacophore. Indeed, CS4566 is hard to interact with Ser209 in the S_3 subsite of caspase-3 active site (Fig. 5A). Therefore, the addition of Asn mimic structure to CS4566 is required for creating more potent and specific inhibitors of caspase-3. Unfortunately, CS4566 had little blocking effect on apoptosis in living cells up to 100 μM (data not shown). The reason is probably little permeability to cell membranes because of its carboxy group in CS4566. We are trying to modify the structure, such as esterification, and to further optimize CS4566 by using SBDD techniques.

In conclusion, our new strategy (COSMOS) for discovery of novel small molecules against target proteins through optimized binding peptides is effective to identify specific inhibitors of caspase-3. CS4566 thus obtained displays inhibition specific for caspase-3 in vitro. So, this compound is an excellent lead that has been searched for a long time in creating of caspase-3 specific inhibitors. Although CS4566 is not yet optimized for caspase-3 inhibitor, understanding how CS4566 interact with caspase-3 might allow us to design optimized small molecules by rational modifications based on its structure in hope of producing potent and selective inhibitors targeting caspase-3. These optimized small molecules might be utilized in the development of new pharmaceuticals for neurodegenerative disorder therapy. Thus, our concept (conversion to small molecules through optimized-peptides strategy) is useful for screening and designing of effective lead small molecules for the creation of new drugs.

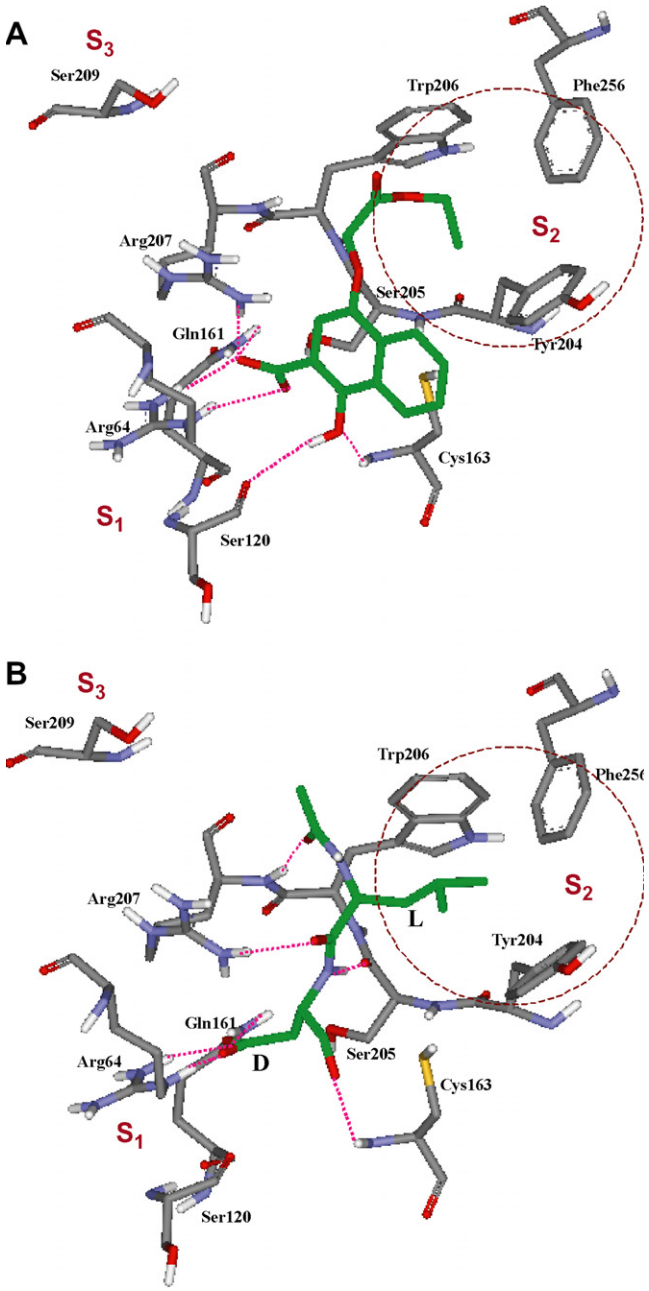

Figure 5. Predicted binding modes of CS4566 and Ac-LD-CHO to caspase-3 active site. The binding modes were obtained from docking simulation. Nitrogen, oxygen, and carbon atoms of the inhibitors are illustrated in blue, red, and green, respectively. Hydrogen bonds are shown as pink dashed lines. Hydrophobic interactions are shown as red dashed circles. S_1, S_2, and S_3 show binding pockets of caspase-3 active site. (A) The binding mode of CS4566 to caspase-3. (B) The binding mode of Ac-LD-CHO to caspase-3. The binding mode of Ac-LD-CHO is configurationally mimicked by that of CS4566 very well.

4. Experimental

4.1. Materials

4-(Ethoxycarbonylmethoxy)-1-hydroxy-2-naphthoic acid (CS4566) and 1,4-dihydroxy-2-naphthoic acid were purchased from Sigma–Aldrich. Recombinant human caspases-3, -7, -8, and -9 were purchased from Calbio-

chem. Ac-DEVD-MCA, Ac-IETD-MCA, and Ac-LEHD-MCA were from Peptide Institute, Inc. (Osaka, Japan).

4.2. Caspase assay and inhibition

The activities of caspases-3 and -7 were measured using Ac-DEVD-MCA as the substrate. The activities of caspases-8 and -9 were measured using Ac-IETD-MCA and Ac-LEHD-MCA, respectively. One unit (manufacturer's definition; the amount of enzyme that cleaves 1 nmol of the peptide substrate per hour) of each recombinant human caspase was preincubated with various concentrations of inhibitors (CS4566 and 1,4-dihydroxy-2-naphthoic acid) in assay buffer (50 mM Hepes, 100 mM NaCl, 10 mM DTT, 0.1 mM EDTA, 10% glycerol, and 0.1% CHAPS, pH 7.4) at 37 °C for 10 min in a 96-well plate (CORNING). Then, caspase substrate was added to each well to a final concentration of 100 μM and the liberation of AMC was continuously monitored at 37 °C using a 96-well plate reader SPECTRA max GEMINI XPS (Molecular Devices) with an excitation wavelength of 390 nm and an emission wavelength of 460 nm.

4.3. Computational molecular modeling and docking

Computational molecular modeling and docking was performed as described previously.[27] Briefly, molecular visualization was carried out in a DS ViewerPro (Accelrys, Inc., San Diego, CA). The coordinate of caspase-3 was obtained from the Protein Data Bank (PDB) (code 1PAU). Water was removed from the PDB file. The crystal structure of caspase-3 includes coordinates of Ac-DEVD-CHO, as an inhibitor. Since it is known that Ac-DEVD-CHO potently inhibits the activity of caspase-3,[21] we constructed the models of the Ac-DEVD-CHO/caspase-3 complex. The geometry of this complex structure was subsequently optimized using the GROMACS program.[31,32] The coordinate of Ac-DEVD-CHO was converted to a GROMACS topology file by the Dundee PRODRG2 Server.[33] Energy minimization of the complex structure using a GROMACS forcefield was performed with the steepest descent algorithm.

Molecular docking was carried out using AutoDock3.0[28] on a COMPAQ Alphastation DS20E (double 833 MHz processors and 1024 MB of memory). The binding free energy scoring function in the AutoDock is based on an empirical function derived by linear regression analysis of a large set of diverse protein–ligand complexes with known inhibition constants. The docking energy grid (grid maps with $60 \times 60 \times 60$ points, grid spacing 0.375 Å) was produced with the AutoGrid program.[34] The inhibitor centers in the complex structures were positioned at the grid center. The Lamarckian genetic algorithm was utilized, and energy evaluations were set at 3×10^6. Each simulation was performed a total of 20 times. Other parameters were default values. Rotational bonds in the inhibitors were assigned with the program AutoTors.[34] The docking data were analyzed using the lowest-energy docking modes.

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