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Noncovalent Tripeptidyl Benzyl- and Cyclohexyl-Amine Inhibitors of the Cysteine Protease Caspase-1

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Potent and noncovalent inhibitors of caspase-1 were produced by incorporating a secondary amine (reduced amide) isostere in place of the conventional electrophile (e.g., aldehyde) that normally confers high potency to cysteine protease inhibitors. Benzyl- or cyclohexylamines produced potent, reversible, and competitive inhibitors that were selective for caspase-1 (e.g., K_i = 47 nM) over caspases 3 and 8 with minimal cytotoxicity. Unlike most cysteine protease inhibitors, these compounds do not react covalently and indiscriminately with thiols.

Introduction

A common strategy for inhibiting some classes of enzymes is to incorporate a reactive electrophile (e.g., aldehyde, ketone, nitrile, chloromethyl ketone, epoxide) in a compound to induce covalent bonding with an enzyme active site nucleophile, such as the side chain of a serine or cysteine residue.[1,2] Such electrophiles can confer up to 10^3- to 10^4-fold enhancements to inhibitor potency of enzyme inhibitors, but also result in indiscriminant interactions with other nucleophiles in vivo, leading to side effects and reduced bioavailability.[1] Cysteine proteases are pivotal enzymes in tumor metastasis (e.g., cathepsin B), osteoporosis (cathepsins K, L), atherosclerosis, inflammation (cathepsin S), aging, apoptosis, neurodegenerative diseases (caspases 3, 8), malaria (falcipain 2 and 3), Chagas disease (cruzipain), and viral infections like the common cold (rhinovirus 3C protease). Many potent inhibitors of cysteine proteases have been developed,[1,2] but few have progressed in clinical trials and none are in the marketplace.[1,2]

Here, we report a series of secondary/tertiary amines without electrophiles that are the first potent and stable noncovalent inhibitors of caspase-1, an important proinflammatory cysteine protease that converts interleukin-1 (IL-1) to the inflammatory cytokine IL-1β. Caspase-1 inhibition reduces formation of IL-1β in vitro and in vivo, with efficacy in inflammatory diseases in animals and humans.[3] No caspase-1 inhibitor has succeeded in clinical trials.[1,3] A few examples of noncovalent inhibitors of the lysosomal cysteine proteases, cathepsins K, S, and L, have been described,[4−6] but there are no examples of stable noncovalent inhibitors for caspases, some of which are key enzyme mediators of cell death. Indeed, the presence of an electrophilic isostere is still thought by most to be essential for producing potent inhibitors of caspases.[1−3] Our strategy to inhibit cysteine proteases avoids covalent bonding to the cysteine, instead obtaining affinity by targeting a prime side binding site. This could be a valuable approach to other inhibitors of cysteine proteases, including cathepsins and other caspases.

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Results and Discussion

Our initial lead compound was the benzylamine **3** (Table 1), featuring a secondary amine at the C-terminus or P1′ position. Reduction of a peptide bond to a secondary amine at a peptidase cleavage site was successfully used in the design and development of aspartic protease inhibitors[7] and has also been applied to inhibitor development for a cysteine protease from *Leishmania mexicana*.[8] Replacement of a peptide moiety, at the prime side binding sites of inhibitors containing secondary amines, with an aryl residue has been suggested to lead to inhibitors of thiol-dependent cathepsins.[4]

The peptidic moiety in **3** (2-Nap-Val-Ala-Asp) was previously identified for selective inhibition of caspase-1,[9] and we have made no attempt in this paper to improve this segment or to make it nonpeptidic. Instead we have focused on investigating alternatives to electrophilic isosteres appended to the C-terminal asparate residue. N-Capped tripeptidyl fragments such as 2Nap-Val-Ala-Asp were prepared (Scheme 1) by starting with Z-Asp(O^{t}Bu)-ol **1**.[10] Deprotection with hydrogen, catalyzed by Pd/C, and reaction of the resultant free amine with Z-Ala-OH, using HBTU[a] for coupling, gave dipeptide alcohol Z-Ala-Asp(OtBu)-ol. Sequential deprotection, followed by coupling of Z-Val-OH then 2-naphthoic acid, yielded alcohol **2**. Oxidation with Dess−Martin reagent, reductive amination with various benzylamines or cyclohexylamine, and deprotection with TFA yielded target compounds **3**−**12** (Scheme 1, Table 1).

With the GOLD program, **3** was docked into the substrate-binding site of caspase-1 (Figure 1). The model predicted that the benzylamine portion of **3** would project into the prime side S1′ binding site of the enzyme. In this binding region, the side chains of enzyme residues Asp288 and His248 were identified as potential partners for polar interactions with inhibitors. It was envisaged that those residues could potentially be targeted

[a]Abbreviations: RT, room temperature; 2-Nap, 2-naphthyl; IL, interleukin; TCP, tritylchloride polystyrene; HBTU, *O*-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoroborate; DIPEA, *N*,*N*-diisopropylethylamine; SPPS, solid phase peptide synthesis; AMC, 7-amino-4-methylcoumarine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio] 1-propanesulfonate; MTT, methylthiazoletetrazolium.

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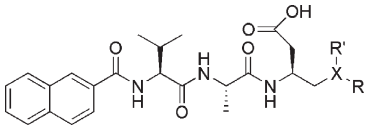
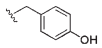
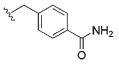
Table 1. Benzylamine Inhibitors of Caspase-1



Compound	R	R'	X	K_i (nM)
3		H	N	600 ± 47
4		H	N	47 ± 7
5		H	N	1200 ± 150
6		H	N	965 ± 76
7		H	N	1370 ± 150
8		H	N	1820 ± 90
9		H	N	209 ± 21
10		H	N	945 ± 99
11		Me	N	1890 ± 260
12		Me	N	128 ± 14
15		H	CH	> 50000

by substituents at the ortho or para positions, respectively, of the benzyl moiety. A hydroxyl substituent was chosen for this task, potentially making a hydrogen bond from **4** and **7** to Asp288 or His248, respectively. These compounds were synthesized and examined for inhibition of human caspase-1. Compound **4** proved to be >10-fold more potent as an inhibitor of caspase-1 than its parent compound **3**. To establish the reason for this increase in potency, the *o*-hydroxy group in **4** was replaced by an *o*-fluorine substitutent (**5**) or an *o*-methoxy group (**6**), resulting in 25- and 20-fold respective decreases in inhibitory potency versus **4**.

The fluorine substituent in **5** retains the electronegative character and steric demand of the hydroxyl group in **4** but removes the H-bond donating property. The methoxy group in **6** removes the H-bond donor but maintains the H-bond accepting oxygen at the ortho position. These assay results indicate that the *o*-hydroxy group acts as a hydrogen bond donor, as predicted from modeling of **4** in the enzyme active site (Figure 1). The model also supported an extended conformation[11] for the inhibitor, orienting the 2-naphthyl residue into the S4 subsite of the enzyme, with Val, Ala, and Asp residues in the S3, S2, and S1 subsites, respectively. The 2-hydroxybenzylamine portion of the molecule was oriented into the S1′ binding pocket. The protonated amine nitrogen atom was found to position between the catalytic residues Cys 285 (N···S = 3.74 Å) and His 237 (N···N = 2.81 Å), with a predicted hydrogen-bonding interaction with His237. The 2-hydroxybenzyl group fills the S1′ subsite, with the 2-OH function directed toward Asp288 and potentially forming a hydrogen bond with the carboxylic acid side chain of this residue (O···O = 2.71 Å).

Compound **7** was synthesized with the intention of targeting His248 through polar interactions exerted by the hydroxy group in the para position. In comparison to unsubstituted **3**, **7** proved to be a less potent caspase-1 inhibitor probably

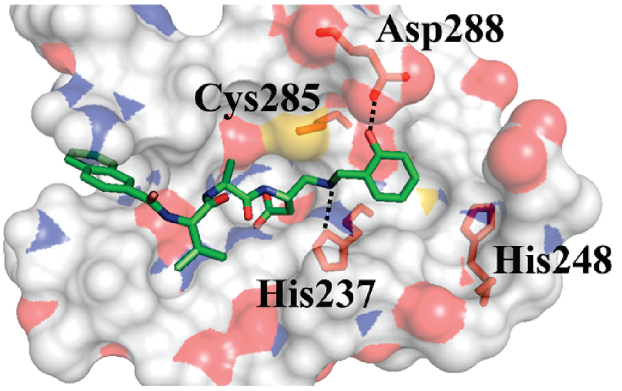
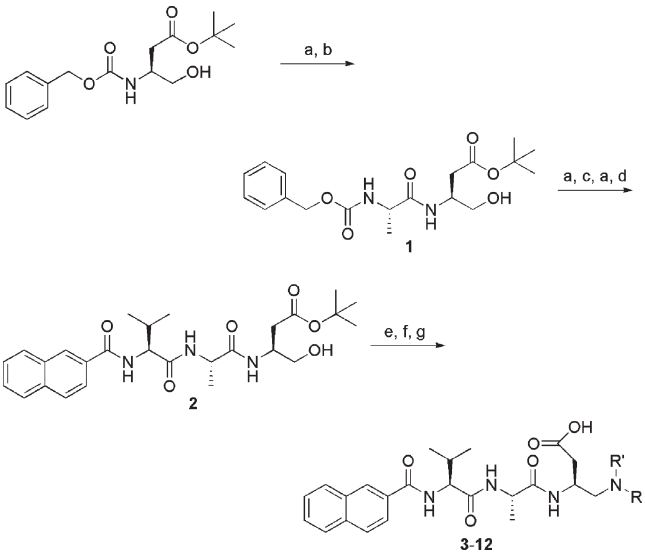


Figure 1. Modeled conformation of **4** docked in the active site of caspase-1 (PDB: 2HBQ). The gray (hydrophobic), blue (basic), and red (acidic) surface represents the active site of the enzyme. The green stick diagram represents ligand **4**. The proposed hydrogen bond is indicated by a dashed line.

Scheme 1. Synthesis of Tripeptide-Derived Amines **3**−**12**[a]

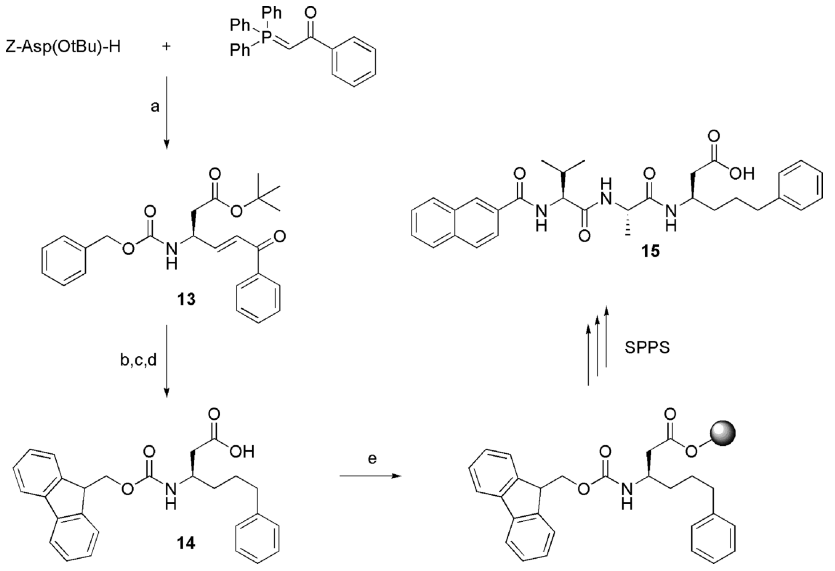


[a] (a) H_2/Pd/C, 1% TFA/EtOAc, RT; (b) Z-Ala-OH, HBTU, DIPEA, DMF, RT; (c) Z-Val-OH, HBTU, DIPEA, DMF, RT; (d) 2-naphthoic acid, HBTU, DIPEA, DMF, RT; (e) Dess−Martin reagent, CH_2Cl_2, RT; (f) RNHR′, $NaBH(OAc)_3$, CH_2Cl_2, RT; (g) TFA/CH_2Cl_2 1:1, RT.

because of a longer distance between the hydroxyl group and the His side chain. Therefore, the hydroxyl substituent in **7** was replaced by a primary amide in **8** to achieve better interactions with the His side chain. If the imidazole of His237 is protonated, it may hydrogen-bond to the primary amide that can serve as an H-bond acceptor or as well a H-bond donor if the His is unprotonated. However, **8** was even less active than **7** as an inhibitor of caspase-1 (Table 1). Thus, His248 seemed to be difficult to target by H-bond forming functional groups.

The docking of **3** in the enzyme had suggested that there is more space in the prime side binding site that could potentially be occupied by extension of the benzyl residue. It was thus decided to prepare **9**, bearing a piperonyl substituent at the amine nitrogen. This residue is a privileged structure in numerous bioactive natural and synthetic products. Indeed, **9** was a 3-fold more potent inhibitor than **3**. Modeling had predicted that the bicyclic benzodioxole component of **9** would fill the P1′ side pocket of caspase-1 very well

Scheme 2. Synthesis of Carba Analogue **15**[a]



[a] (a) EtOAc, reflux; (b) H_2 (40 psi), Pd/C, 3% TFA/ethyl acetate, RT; (c) TFA, RT; (d) FmocOSu, $NaHCO_3$, THF/H_2O 1:1, 4 °C → RT; (e) TCP resin, DMF, RT.

(Supporting Information). The CH−C_{ipso}−CH_2−NH dihedral angle in the piperonyl derivative **9** suggests that the piperonyl ring is oriented perpendicularly to the plane of the benzene ring of **4**. This leads to the conclusion that it may be difficult to structurally combine the space-filling properties of bicyclic moieties with an H-bond donor that targets Asp 288. Thus, when the aromatic benzyl moiety was replaced by cyclohexyl, as in **10**, there was a slight decrease in inhibitory potency compared to **3**.

Attention was next focused on the potential impact of the basic nitrogen on the inhibitory potency. The tertiary methylamine **11** proved to be 3 times less potent as an inhibitor than the corresponding secondary amine **3**. In the case of the cyclohexylamines **10** and **12**, the tertiary methylamine **12** was more active than its secondary amine counterpart **10**. The importance of the amino functionality to the potency of these inhibitors is highlighted by the carba analogue **15**, which was synthesized from the aldehyde Z-Asp(O^{t}Bu)-H[10] (Scheme 2). A Wittig reaction of this aldehyde with 1-phenyl-2-(triphenyl-λ^5-phosphanylidene)ethanone,[12] a stabilized phosphorus ylide, gave intermediate enone **13** that could be completely reduced and N-deprotected with hydrogen catalyzed by Pd/C. Deprotection with TFA and introduction of the Fmoc protecting group via Fmoc-OSu gave **14**. Compound **14** was attached to TCP resin and the target compound **15** assembled via standard solid phase peptide synthesis (Supporting Information). Carba analogue **15** was a very poor inhibitor of caspase-1, suggesting that the amino group of these inhibitors forms crucial polar contacts with the enzyme. None of the compounds described herein showed any detectable cytotoxicity, at the concentrations tested (≤200 μM), in an MTT assay performed with HT29 human cells (see Experimental Methods).

All of the structure−activity relationships interpreted above are based on the assumption that every compound binds in the substrate-binding active site of the enzyme. To establish that the benzylamine compounds were indeed directed toward the active site, we needed to demonstrate that inhibition was competitive with substrate. To facilitate this analysis, the inhibition by **4** was investigated in more detail at varying substrate concentrations. Data were analyzed using a Cornish−Bowden plot,[13] which revealed a set of approximately parallel lines (Figure 2). This confirms a competitive binding and inhibition mode, as expected for such substrate-like inhibitors.[13]

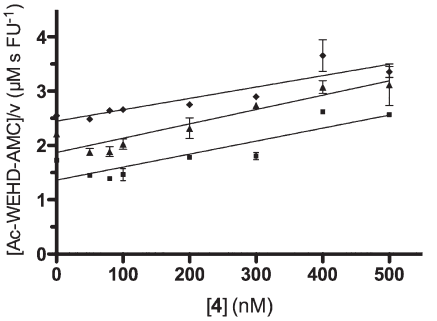
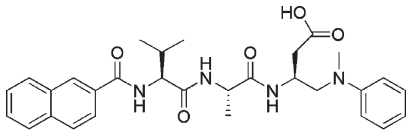


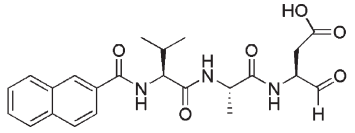
Figure 2. Cornish−Bowden plot showing competitive inhibition of caspase-1 by **4**. The steady-state rates for caspase-1 catalyzed hydrolysis of Ac-Trp-Glu-His-Asp-AMC were measured in the presence of different concentrations of inhibitor **4** ([**4**] = 0, 50, 80, 100, 200, 300, 400, 500 nM). The substrate concentrations were 8 μM (squares), 20 μM (triangles), and 30 μM (lozenges).

The tripeptide-derived amines are slow-binding inhibitors for caspase-1. All K_i values were determined in preincubation assays (Supporting Information). As an example, **4** was also evaluated in an assay in which the reaction was started by adding enzyme to follow the binding kinetics (Supporting Information). This led to confirmation of a one-step mechanism for the enzyme−inhibitor interaction, with a second-order rate rate constant for formation of the enzyme−inhibitor complex of k_{on} = 1389 M^{-1} s^{-1} and a first-order rate constant describing the decay of the complex k_{off} = 6.5 × 10^{-5} s^{-1}.[14] Compound **4** was also evaluated for caspase selectivity by testing against caspases 3 and 8 (Figure S2 of Supporting Information). No inhibition of caspase-3 was observed up to micromolar concentrations, while some inhibition of caspase-8 was detected at high micromolar concentrations. This selectivity for caspase-1 is undoubtedly a result of the naphthyl group being at P4 in **4** and has little to do with the benzylamine isostere.

Chart 1. Aniline Derivative **16** and Corresponding Aldehyde **17**



16



17

With regard to inhibitor stability, the benzylamines described here represent an important advance over covalent and noncovalent inhibitors previously described for cysteine proteases. Covalent inhibitors that possess a reactive functional group for interaction with the active site of the enzyme are generally prone to racemization. For example, the well-known aldehyde inhibitor of caspase-1, Ac-Tyr-Val-Ala-Asp-H, readily racemizes in solution at room temperature.[9] Noncovalent arylamines have been reported as inhibitors of cathepsins K and S,[4] but we have found that such compounds are highly susceptible to oxidation, forming traces of aldehyde intermediates. The presence of even trace amounts of aldehyde in noncovalent arylamines can confer the impression of potent inhibition and thus seriously compromise their assessment as potent and noncovalent inhibitors, as found for azide inhibitors of cysteine proteases.[15] For example, when **16** (Chart 1) was allowed to stand for a few weeks at room temperature, its apparent potency against caspase-1 increased dramatically. This is attributed to the presence of trace amounts (1−2%) of the potent aldehyde inhibitor **17** (IC_{50} = 0.2 nM) detected after aging the samples for 2 weeks (Supporting Information). The aldehyde was produced by oxidation of the aromatic amine of **16**.[16] A series of arylethylamine inhibitors of caspase-1 were similarly all found to increase in inhibitory potency over time, and all showed the formation of traces of **17** in aged samples when analyzed by LCMS (data not shown). By contrast, no aldehyde was detected after storing **4** for < 1 year at room temperature.

Conclusion

Covalent interactions made between cysteine proteases and their prospective inhibitors have previously been identified by some as a major impediment to successful clinical development of cysteine protease inhibitors.[1,17] However, progress toward the development of noncovalent inhibitors of cysteine proteases, which by contrast do not indiscriminately interact with numerous nucleophiles in vivo, has been very slow. The present study has contributed one possible solution to this problem. By incorporating a secondary amine (reduced amide) isostere into putative inhibitors, with additional focus on making key interactions between inhibitor and the prime side binding sites of the enzyme, this study has produced potent, reversible, and noncovalent inhibition of caspase-1. This strategy of avoiding electrophilic isosteres could be applied to developing inhibitors of other important cysteine proteases, with the caveat that some arylamines, vide infra, are unstable and convert to potent aldehyde contaminants that can compromise enzyme inhibition results. We believe that this field should progress from cysteine protease inhibitors bearing electrophilic warheads to the design of noncovalent inhibitors, a step that occurred successfully some years ago for serine protease inhibitors and has since translated effectively into clinical trials.[1]

Experimental Methods

Synthesis of Tripeptidylamine-Based Inhibitors of Caspase-1. The synthesis of **4** only is described here, while the synthesis of all other compounds is reported in the Supporting Information.

(*S*)-3-((*S*)-2-((*S*)-2-(2-Naphthamido)-3-methylbutanamido)-propanamido)-4-(2-hydroxy)benzylaminobutanoic Acid, 4. This is also a general procedure used for the syntheses of tripeptidylamines **3**−**12**. The tetrapeptide aldehyde (0.05 g, 0.1 mmol) was dissolved in dichloromethane (5 mL) and benzylamine (0.013 mL, 0.12 mmol) (or an equivalent amount of substituted benzylamine or cyclohexylamine) was added followed by solid sodium triacetoxyborohydride (0.11 g, 0.5 mmol). After the mixture was stirred at room temperature for 2 h, the solvent was removed in vacuo and the residue suspended in 1 N NaOH and extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with saturated NaCl (10 mL), dried over $MgSO_4$, and evaporated to yield an oily colorless residue which was taken up in CH_2Cl_2/TFA (1:1, 10 mL). After the mixture was stirred for 2 h, the volatiles were removed in the N_2 stream. The oily material was subjected to purification by preparative HPLC. The product-containing fractions (monitored by ESMS) were combined and lyophilized to give the pure final product **4**, as the trifluoroacetate salt, in 30% overall yield. ^{1}H NMR (600 MHz, DMSO-d_6) δ in ppm = 0.95 (^3J = 6.5 Hz, 3H, Val-CH_3); 0.96 (^3J = 6.5 Hz, 3H, Val-CH_3); 1.23 (d, ^3J = 7.0 Hz, 3H, Ala-CH_3); 2.10−2.19 (m, 1H, Val-C_βH); 2.53 (d, ^3J = 6.4 Hz, Asp-CH_2); 3.08−3.15 (m, 2H, CHCH_2NH); 4.06−4.15 (m, 2H, PhCH_2NH); 4.19−4.25 (m, 1H, Ala-CH); 4.26−4.37 (m, 2H, Asp-CH, Val-C_αH); 6.80−6.85 (m, 1H, Ph-5-H); 6.90 (br d, ^3J = 8.1 Hz, 1H, Ph-3-H); 7.20−7.25 (m, 1H, Ph-4-H); 7.31 (br d, 1H, 7.5 Hz, Ph-6-H); 7.57−7.64 (m, 2H, Nap-6-H, 7-H); 7.92−8.00 (m, 4H, Nap-3-H, 4-H, 5-H, Asp-NH); 8.03 (br d, ^3J = 7.9 Hz, 1H, Nap-8-H); 8.25 (d, ^3J = 6.8 Hz, 1H, Ala-NH); 8.42 (d, ^3J = 8.2 Hz, Val-NH); 8.50 (br s, 1H, Nap-1-H); 10.21 (br s, OH). ^{13}C NMR (150 MHz, DMSO-d_6) δ in ppm = 17.63 (Ala-CH_3); 18.95 (Val-CH_3); 19.32 (Val-CH_3); 30.14 ((CH_3)$_2$-CH); 36.44 (CH_2COOH); 43.45 (Asp-CH); 46.00 (PhCH_2NH); 48.61 (Ala-CH); 49.14 (CHCH_2NH); 59.14 (Val-CH); 115.29 (Ph-3-C); 117.88 (Ph-1-C); 119.08 (Ph-5-C); 124.47, 126.72, 127.61, 127.64, 127.75, 127.78, 128.85, 130.55, 131.43 (CH_{arom}); 131.50, 132.08, 134.19 (C_{arom}); 156.06 (Ph-2-C); 166.75 (Nap-CO); 171.04 (Val-CO); 171.66 (COOH); 172.46 (Ala-CO). HR-ESMS calcd for $C_{30}H_{37}N_4O_6$ $[M + H]^+$ = 549.2713, found 549.2701. Analytical HPLC 100% A to 100% B in 20 min: % purity = 98.3%, t_R = 13.73 min.

Methods for Molecular Docking. The crystal structure coordinates of caspase-1 were obtained from PDB entry 2HBQ.[18] Coordinates for peptidic ligands were developed using the Sybyl sketch module and further refined by minimization using a conjugate gradient method[19] and molecular dynamics using the Tripos force field.[20] Docking of ligands was performed using Gold, version 3.2,[21] with default docking settings. The active site of the enzyme was defined by taking Ser339 at the center of the active site with a 12 Å radius. Ligands were docked in 30 independent experiments (each involving 100 structures and up to 100 000 operations). Mutations, migration, and operator weights for crossover were set to 95, 10, and 95, respectively. To allow poor nonbonded contacts, a maximum distance of 5 Å was set between hydrogen donor and fitting points. To speed up calculations, docking was stopped when rmsd = 1.5 Å between the top three solutions. Docking was done without any bonding constraints between the protein and the ligand. The best-docked conformation of the ligand was identified manually by considering the highly conserved orientation with maximum number of interactions within the active site.

Inhibition Experiments and Inhibition Kinetics of Compound 4. Recombinant human caspase-1 (Calbiochem) was diluted with 700 μL of buffer (identical to the assay medium except that it lacks DMSO), divided into 70 μL aliquots, and stored at −80 °C. A 1:20 dilution of these aliquots was used in the assays. Enzyme activities were calculated from kinetic measurements by fluorimetric detection of the product 7-amino-4-methylcoumarine (AMC) at 30 °C in a plate reader (Polarstar BMG Labtech) using black 96-well plates (final volume of 200 μL). The wavelengths for excitation and emission were 380 and 460 nm, respectively. A 2 mM stock solution of the fluorogenic substrate Ac-Trp-Glu-His-Asp-AMC (Calbiochem) was prepared in DMSO. The final concentration was 5 μM. The assay medium consisted of 50 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 10% glycerol, 0.1% CHAPS, 1% DMSO. Stock solutions of the inhibitors (each 5 mM) were prepared in DMSO.

Inhibition by **4** of caspases 3 and 8 is described in the Supporting Information (Figure S2).

MTT Cytotoxicity Assay. The cytotoxicity of the caspase-1 inhibitors was tested against HT29 colon cancer cells. Cells were plated in a 96-well plate at 1×10^5 cells/well, using RPMI 1640 medium, and left to adhere overnight at 37 °C. The following day, cells were treated with various drug concentrations and left to incubate for 24 h at 37 °C. MTT dissolved in RPMI 1640 media at 1 μg/mL was added to the cells (100 μL/well) and incubated for 1 h at 37 °C to allow for MTT to be metabolized. Cells were then lysed and crystals dissolved using isopropanol. Measurements were taken using a Biotek Powerwave XS microwell plate reader at 570 nm.

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Supporting Information Available: Synthetic procedures and characterization data of all compounds, docking images for **3** and **9**, inhibition kinetics of **4** and demonstration of selectivity over caspases 3 and 8, and experimental evidence for the autoxidation of **16**. This material is available free of charge via the Internet at http://pubs.acs.org.

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