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Tethering identifies fragment that yields potent inhibitors of human caspase-1☆

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Abstract—Disulfide Tethering® was applied to the active site of human caspase-1, resulting in the discovery of a novel, tricyclic molecular fragment that selectively binds in S4. This fragment was developed into a class of potent inhibitors of human caspase-1. Several key analogues determined the optimal distance of the tricycle from the catalytic residues, the relative importance of various features of the tricycle, and the importance of the linker.

The caspases (cysteinyl aspartate-specific proteases) are a family of endopeptidases that share a preference for an aspartic acid in the substrate P1 position.[2,3] Caspase-1 (interleukin-1β converting enzyme, ICE) carries out cytokine processing that results in inflammatory responses, and this processing is believed to play a key biological role for the enzyme.[4,5] There are two known endogenous substrates for caspase-1: pro-interleukin-1β (pro-IL-1β) and pro-interleukin-18 (pro-IL-18). IL-1β is a proinflammatory cytokine that is released by peripheral blood mononuclear cells in response to inflammatory stimuli. This cellular release of IL-1β requires processing of pro-IL-1β by caspase-1.[6] Inhibitors of caspase-1 might therefore attenuate the inflammatory processes that occur in various diseases.

Tethering identifies small molecular fragments that bind to a specific site on a protein surface and can form disulfide bonds with a cysteine residue near that site.[7,8] Using Tethering, we discovered a tricyclic molecular fragment that binds in the S4 region of caspase-1 (Fig. 1).[9] Having quickly converted this fragment into a promising inhibitor, we herein report some of our efforts to develop a more advanced lead compound.

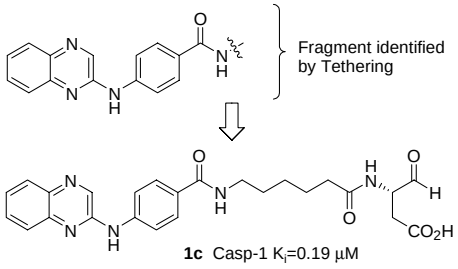

Figure 1. Initial hit derived from Tethering.

Our initial hit **1c** was assembled by linking the tricyclic fragment to a methylene scaffold bearing an aspartyl aldehyde, a well-known P1 moiety for caspase inhibitors.[10]

The length of the tether used in the disulfide formation led us to expect that the optimal length of our inhibitors would require a 6-aminohexanoic acid linker between the tricyclic fragment and the aspartyl aldehyde.[9] This length corresponds to a direct atom-for-atom replacement of the Tethering disulfide linkage with a methylene chain. Indeed, a study of unsubstituted, flexible linkers

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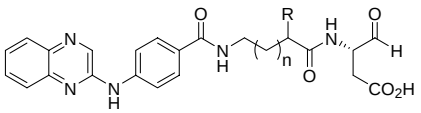
Table 1. P2 groups and length dependence



Compound	*n*	R	K_i (μM)
1a	1	H	5.2
1b	2	H	2.8
1c	3	H	0.190
1d	4	H	2.6
2	3	2-Thienyl	0.005
3	3	Ethyl	0.016
4	3	Phenyl	0.005
5	3	2-Fluorophenyl	0.008

(compounds **1a**–**d**, Table 1) demonstrated that the K_i of the inhibitors was strongly dependent on length, with 6-aminohexanoic acid giving the most potent inhibitor (**1c**).

We had previously found that caspase-3 inhibitors bearing a hydrophobic P2 substituent often displayed good potency against caspase-1.[10] We therefore attached our P4 tricycle to a set of flexible linkers with various hydrophobic P2 groups (Table 1). As anticipated, the presence of a P2 group greatly improved potency. Although several compounds displayed low nanomolar potency, concerns about the number of rotatable bonds led us to examine a series of more constrained linkers (Chart 1, Table 2). The synthesis of these linkers has been reported elsewhere,[8] except for the peptidyl analogue **15**, which was assembled using standard solid-phase peptide techniques. Several linkers produced high-affinity inhibitors, with the pyridyl-linked **11** being the

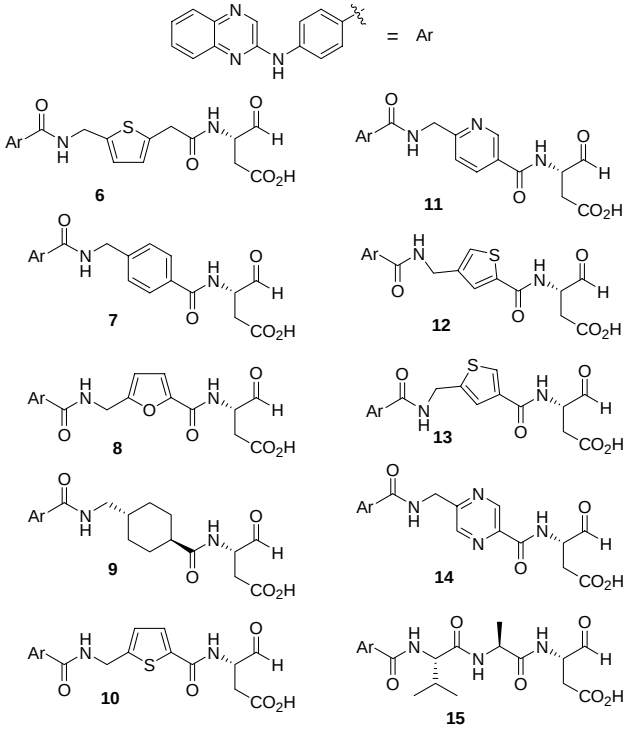


Chart 1. Rigid linkers.

Table 2. Variation of linker

Compound	Casp-1 K_i (μM)
6	0.12
7	0.10
8	2.10
9	1.95
10	0.035
11	0.005
12	0.26
13	0.12
14	1.0
15	0.004
Z-VAD-CHO	0.008

most potent non-peptide. Pyridyl and benzenoid linkers, similar to those in **7** and **11**, had previously been incorporated into a series of reversible caspase inhibitors by Cytovia[11] and into a series of irreversible inhibitors by Vertex.[12] The thiophene-linked compound **10** was 9-fold less potent at 35 nM.

We next turned our attention to the tricycle in order to establish which elements are important for inhibition and to improve the physical properties of the inhibitors (Chart 2, Table 3) For this study, we chose two linkers, 6-aminohexanoic acid and the thiophene-substituted linker in compound **2**. Truncating the quinoxaline system (**16a**,**b**) reduced the potency, which could be partially restored by placing a chlorine at the pyrazine 6-position (**17b**). Replacing the bridging NH with an oxygen moderately reduced the potency (**18a**,**b**). Methyl groups are tolerated at several positions with only modest changes in potency (**19a**,**b** and **20b**). The reverse amide (**21a**) analogue of our initial lead (**1a**) had an approximately 10-fold reduced potency.

The syntheses of the P4 groups are summarized below (Scheme 2). With the exception of the reverse amide **21a**, all P4 analogues were synthesized as carboxylic acids and then elaborated into the full inhibitors using the resin chemistry shown in Scheme 1. Similar resin chemistry to prepare aldehydes has been previously

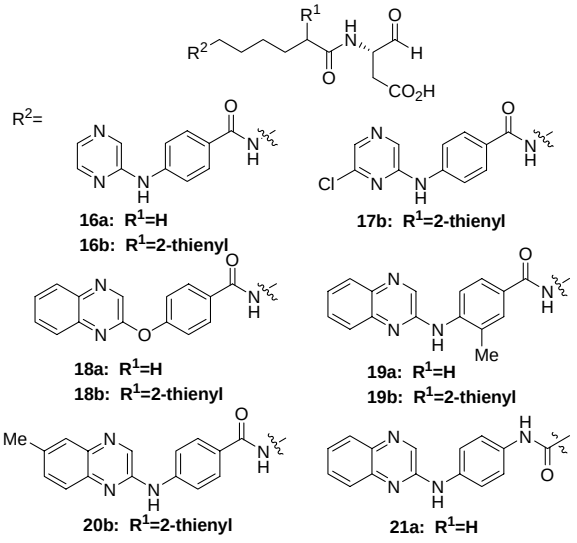
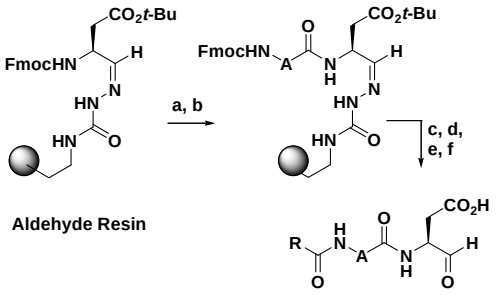
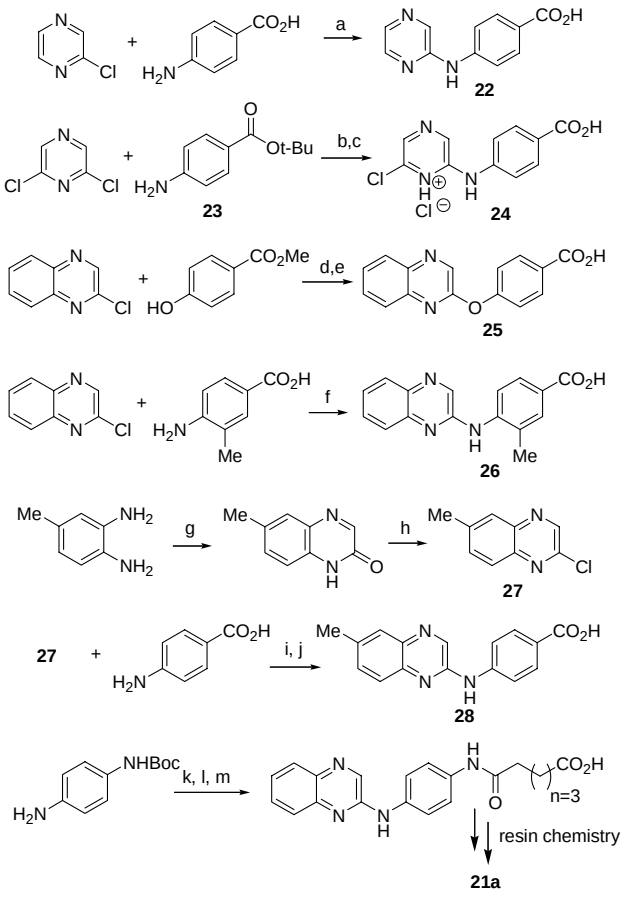


Chart 2. Variation of P4 group.



Scheme 1. Inhibitor syntheses. Reagents: (a) 20% piperidine/DMF; (b) FMOC-NH-A-CO_2H; (c) 20% piperidine/DMF; (d) RCO_2H, PyBoP, DIEA; (e) TFA/AcOH/TFA, MeCHO; (f) CH_2Cl_2, TFA.



Scheme 2. Syntheses of P4 groups. Reagents and conditions: (a) HCl, H_2O, 100 °C, 1.5 h, 8%; (b) NaH, DMSO, rt, 9 days, 22%; (c) HCl, dioxane, rt, 44%; (d) NaH, DMSO, 80 °C, 16 h, 85%; (e) LiOH, H_2O, dioxane, 93%; (f) HCl, H_2O, 100 °C, 1 h, 56%; (g) glyoxylic acid(aq), MeOH, rt, 42%; (h) $POCl_3$, PCl_5, 110 °C, 2.5 h,89%; (i) HCl, MeOH, 90 °C, 46%; (j) LiOH, H_2O, dioxane, 60%; (k) **17**, EtOH, Δ; (l) TFA; (m) adipic anhydride.

reported by groups at Corvas,[13] Idun,[14] and Sunesis.[8] The pyrazine **22** was prepared by condensing 4-aminobenzoic acid with chloropyrazine. The 6-chloro analogue thereof (**24**) was prepared as the hydrochloride salt, using an S_NAr reaction of **23** with 2,6-dichloropyrazine, followed by cleavage of the *tert*-butyl ester. The oxygen-bridged tricyclic analogue **25** was prepared by reacting methyl 4-hydroxybenzoate with 2-chloroquinoxaline, followed by hydrolysis of the ester. The 6-methyl quinoxaline **28** was prepared from the chloroquinoxaline **27** by treating **27** with 4-aminobenzoic acid in acidic methanol to give the methyl ester of **28**, which was then hydrolyzed to provide the acid **28**. The synthesis of the reverse amide **21a** was accomplished by first forming the tricyclic amine, followed by reaction with adipic anhydride, then coupling of the resulting carboxylic acid onto the resin-supported aldehyde.

Unfortunately, none of the P4 analogues in Table 3 showed potency greater than that of the original tricycle. Nevertheless, the apparent specificity of the original tricycle for the S4 site demonstrates the power of Tethering to discover molecular fragments that form a tight and selective fit with a protein surface.

Some of our more potent analogues showed low micromolar cell activity in a peripheral blood mononuclear cell (PBMC) assay measuring the release of IL-1β (Table 3).[15] Compound **1c**, having a completely unsubstituted and flexible linker, in fact displayed the best cell activity in the series at 4.2 μM. This activity was approximately equal to that of the peptide aldehyde Z-YVAD-CHO we used as a standard. As a control, for compounds tested in the cell assay we also measured IL-6 production and found in all cases that it was unaffected at inhibitor concentrations up to 200 μM.

Crystal structures of **11** and **18b** bound to caspase-1 showed the tricycle in two different binding modes (Fig. 2). The tricycle of compound **11** sits in a lower groove in the S4 pocket, whereas the tricycle in compound **18b** sits in an upper groove. The fact that compound **11** was more potent suggests that the lower binding mode is more energetically preferred. This hypothesis is further supported by crystal structures we recently disclosed in another publication, in which the highest potency analogues all displayed the lower binding mode.[9]

Space-filling models show a remarkably tight filling of the space within S4. The guanidine of Arg383 sits over the phenyl ring of the tricycle, possibly forming a π-charge interaction. Compound **11** forms a hydrogen bond between the pyridyl nitrogen and the backbone nitrogen of Arg341. This hydrogen bond presumably contributes to the high potency of compound **11**.

Table 3. Variation of P4 group

Compound	R^1	Casp-1 K_i (μM)	IL-1β EC_{50} (μM)
1c	H	0.19	4.2
2	2-Thienyl	0.007	20.5
16a	H	37	>200
16b	2-Thienyl	0.10	20.0
17b	2-Thienyl	0.054	n.d.
18a	H	2.4	27.0
18b	2-Thienyl	0.043	12.4
19a	H	0.32	7.7
19b	2-Thienyl	0.004	7.7
20b	2-Thienyl	0.005	n.d.
21a	H	2.1	n.d.
Z-YVAD-CHO	—	0.18	3.9

n.d., not determined.

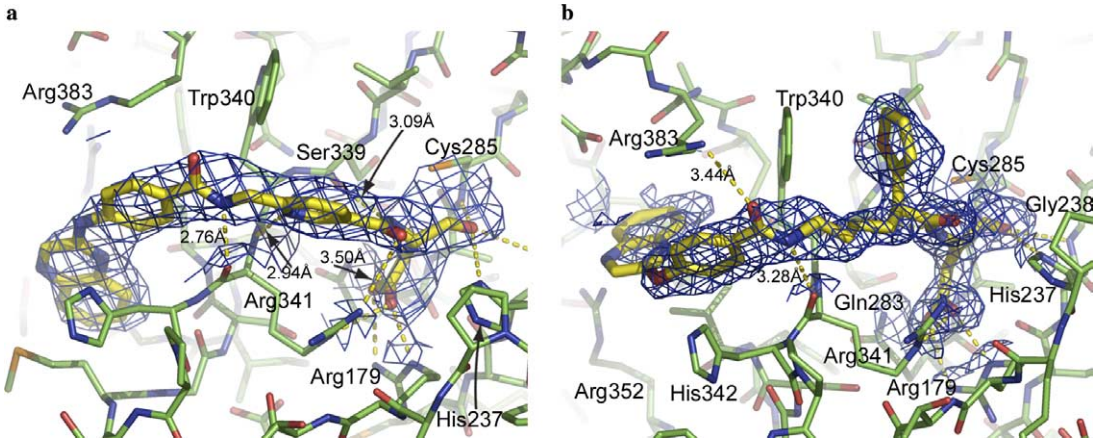


Figure 2. (a) Crystal structure of compound **11** (PDB ID code: 1rww). In yellow are hydrogen bonds. (b) Crystal structure of **18b** (PDB ID code: 1rwx). A $2F_o - F_c$ electron density map within a 3.5-Å distance of each inhibitor, contoured at the 1σ level, is shown as a blue mesh.

In conclusion, we have used a novel tricyclic molecular fragment, provided by Tethering, to develop a series of potent caspase-1 inhibitors. Analogue studies of the tricycle demonstrated the importance of its structural features and multiple co-crystal structures showed the importance of the linker structure.

Acknowledgments

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