"Click" synthesis of small-molecule inhibitors targeting caspases†‡

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A panel of 198 P_4-diversified aldehyde (reversible) and vinyl sulfone (irreversible) inhibitors is successfully synthesized *via* an efficient "click chemistry" platform and directly screened against caspase-3 and -7 for inhibition.

Caspases belong to a class of cysteine proteases which play important roles in the regulation of apoptotic cell death and inflammatory responses.[1] Potent and selective caspase inhibitors may therefore be used to cure major human diseases such as Alzheimer's disease, cancer and arthritis. Over the past decade, a variety of reversible and irreversible inhibitors targeting various classes of cysteine proteases, including caspases, have been developed.[2] These inhibitors, both peptide- and non-peptide-based, usually contain an electrophilic 'warhead' derived from aldehydes, ketones, vinyl sulfones and heterocycles, which reacts with the catalytic cysteine residue present in all caspases. Amongst the different types of warheads, those that make use of aldehydes and vinyl sulfones (VS), as reversible and irreversible caspase inhibitors respectively, have been well documented (Fig. 1a, top).[2] Caspases exclusively recognize and cleave substrates that possess an aspartic acid residue at the P_1 position. In addition, the P_4 position is often the key determinant that confers both strong binding and selectivity among different caspases. The P_2 and P_3 positions in a caspase substrate, on the other hand, are known to play a less significant part in binding to the enzyme. Consequently, most peptide-based, caspase inhibitors contain a tetrapeptide sequence, P_4-P_3-P_2-Asp, coupled to a suitable warhead.[3] By varying primarily the P_4 residue, potent and selective inhibitors may be developed that target only a subset of caspases. Non-peptide-based small molecule caspase inhibitors, on the other hand, are better therapeutic agents, due to their desirable pharmacokinetic properties. In a recent example by Choong and co-workers, a series of non-peptidic inhibitors of caspase-3 was identified using a fragment-based ligand discovery technology known as "extended tethering".[4] Using this method, the authors were able to discover highly potent, non-peptide-based caspase inhibitors by "tethering" together two weakly binding fragments *via* a suitable linker (Fig. 1a, bottom). This strategy was reminiscent of the better-known fragment-based assembly approach which typically relies on advanced NMR techniques.[5] In this case, weak binders of the enzyme were rapidly identified using state-of-the-art mass spectrometric techniques.[4] Subsequent optimizations of the initial "hits" gave rise to **A** (Fig. 1a, bottom). With an aspartic acid-containing aldehyde warhead at the P_1 position, a pyrimidine linker occupying the P_2 and P_3 positions, and a salicylic acid sulfonamide at the P_4 position, this non-peptide inhibitor was found to display a high inhibitory potency against caspase-3 (k_i = 0.02 μM). Inspired by this work, we aim to develop a simpler, more efficient, miniaturized strategy, aided by click chemistry, for high-throughput synthesis of caspase inhibitors containing such novel, small-molecule pharmacophores.

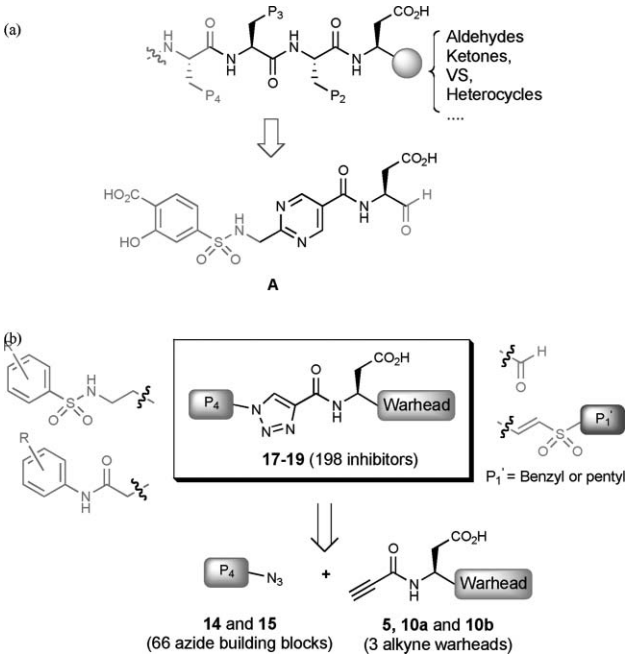

Fig. 1 (a) Structure of peptide- and non-peptide-based caspase inhibitors; (b) "Click" synthesis of a panel of 198 small molecule caspase inhibitors.

Click chemistry is a concept coined by Sharpless *et al.*[6] It refers to several classes of chemical transformations which enable the modular and highly efficient assembly of building blocks under mild, assay-ready, aqueous conditions. The Cu(I)-catalyzed, 1,3-dipolar cycloaddition between an azide and an alkyne is one of the best studied click chemistry reactions,[7] and has thus far been adopted by various research groups for high-throughput discovery of enzyme inhibitors against HIV-1 protease, SARS 3CL protease, α-fucosidase, transferases, protein tyrosine phosphatases (PTPs), matrix metalloproteases (MMPs) and others.[8] Herein, we report the first "click" library of inhibitors for caspases. As shown in Fig. 1b, a panel of P_4-diversified caspase inhibitors containing

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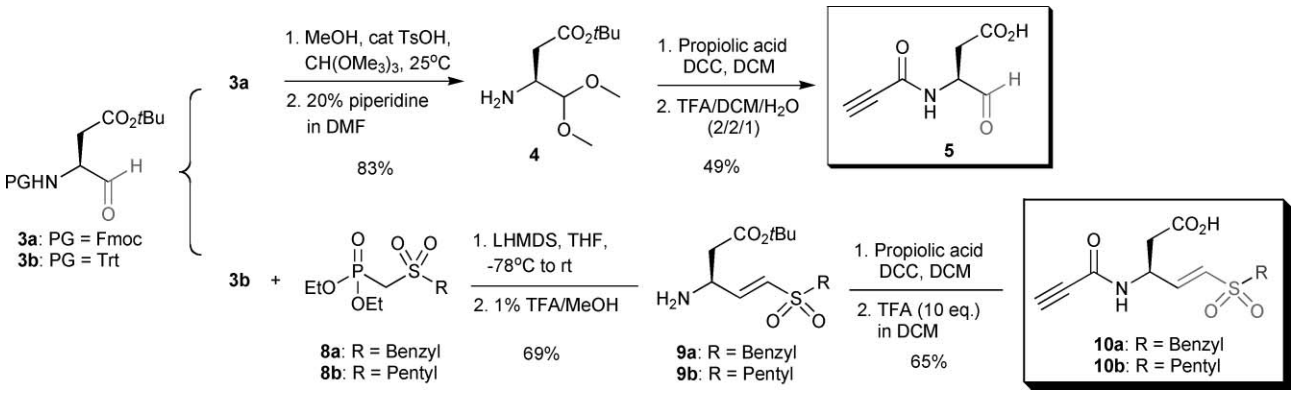
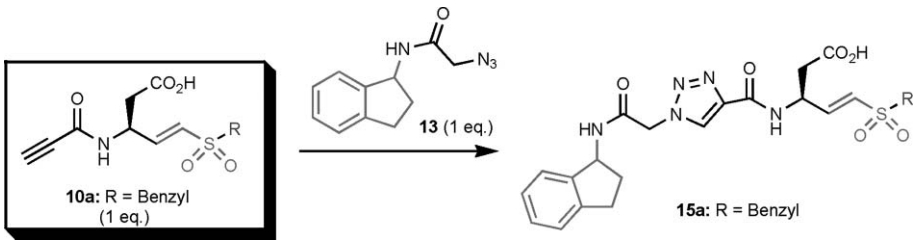


Scheme 1 Synthesis of aspartic acid-containing alkyne warheads.



Scheme 2 Model reaction for click chemistry optimization.

either aldehyde (reversible) or VS (irreversible) warheads were readily synthesized in 384-well microplates, and directly screened against caspase-3 and -7. Our inhibitor design encompasses the following features: (1) the absolute requirement of an aspartic acid residue at the P_1 position, (2) a benzyl or pentyl group at the P_1' position of the VS inhibitors. These groups were previously shown to improve inhibitor potency towards caspase-3 and -7,[9] (3) a triazole linker (product of click chemistry) occupying the P_2 and P_3 position, and (4) a diverse library of aromatic amide and sulfonamide building blocks at the P_4 position. Aromatic/hydrophobic groups are known to preferably occupy the S_4 pockets of caspase-3 and -7.[4] The application of click chemistry in our approach is essential due to several reasons. First, the mild nature of click chemistry excludes the need for any base or nucleophile, making it ideal for the assembly of electrophilic/reactive aldehyde and vinyl sulfone warheads.[2,9] Second, since the P_2 and P_3 positions play a less significant role to the overall potency and selectivity of caspase inhibitors,[1] replacing them with a heterocyclic triazole linker (which resembles the pyrimidine linker in **A**[4]) may impart potential H-bond interactions with the enzyme.[10] Last, the use of "click" assembly allows the facile installation of a variety of diverse P_4 groups onto the warheads using readily available azide building blocks in a miniaturized and parallel fashion (*e.g.* 384-well plates).

The detailed synthesis of the aspartic acid-containing alkyne warheads is shown in Scheme 1. Starting from a Fmoc-protected aspartic acid, the corresponding aldehyde **3a** was obtained from a two-step reduction followed by Swern oxidation as reported.[11] After protection of the aldehyde using trimethyl orthoformate and deprotection of Fmoc, the alkyne handle was installed using DCC coupling with propiolic acid. Removal of protecting groups in the last step afforded the aspartic-containing alkyne aldehyde warhead **5** in good yields. The synthesis of the irreversible VS warheads was carried out using a similar aldehyde intermediate **3b** except that the protecting group was changed to a trityl group. We took into consideration that the VS scaffold was sensitive to nucleophiles such as piperidine.[9a] Hence, the use of an acid-labile trityl group instead of Fmoc avoided potential problems. This trityl-protected aldehyde was then reacted with the respective benzyl and pentyl sulfones **8** through Horner–Wadsworth–Emmons condensation to form the VS scaffold **9**.[9a] Subsequently, the final VS warheads **10** were obtained with the attachment of propiolic acid and deprotection of the *tert*-butyl ester.

Initial attempts to investigate the applicability of click chemistry on our inhibitor library were carried out in model studies using the VS benzyl warhead **10a** and azide **13** (Scheme 2). A variety of click chemistry conditions were investigated (Table 1). It appeared that the triazole formation between alkyne warhead **10a** and azide **13** was highly sensitive to the catalyst, additives and solvents used. The reaction efficiency varied greatly across different conditions. For instance, the use of CuBr and Cu/C catalyst (entries 8 and 9) showed no product formation even after 4 days, while the use of CuI catalyst (entries 4–7) showed a complete reaction in 2 days. However, the need for a nucleophilic base under these conditions might have also resulted in the formation of by-products (presumably due to reaction with the electrophilic VS warhead[9a]), hence giving rise to the observed poor purities. Fortunately, entry 2 (with $CuSO_4$ and sodium ascorbate as catalysts and DCM–H_2O as cosolvent) gave rise to the desired product with excellent yield and purity (in most cases the reaction was complete in 1 day and only the desired product was observed; see ESI). Therefore, we used these conditions as our general optimized procedure for all subsequent "click" assembly of inhibitors.

Next, we synthesized the 198 caspase inhibitors (66 azides × 3 alkynes) in a 384-well plate. Common laboratory apparatus (multi-channel pipettes and a bench-top shaker) was all that was needed

Table 1 Optimization conditions of click chemistry

Entry	Catalyst	Base/additive	Solvent	Time	% Yield[a]	% Purity[a]
1	$CuSO_4 \cdot 5H_2O$ (0.05 eq.)	Na Asc (0.2 eq.)	tBuOH : H_2O 1 : 1	>4 d	50	50
2	$CuSO_4 \cdot 5H_2O$ (0.05 eq.)	Na Asc (0.2 eq.)	DCM : H_2O 1 : 1	1 d	>95	>95
3	$CuSO_4 \cdot 5H_2O$ (0.05 eq.)	Na Asc (0.2 eq.)	DMSO : H_2O 1 : 1	>2 d	60	60
4	CuI (1 eq.)	DIPEA (2 eq.)	tBuOH : H_2O 1 : 1	2 d	>95	90
5	CuI (1 eq.)	DIPEA (2 eq.)	DMSO : H_2O 1 : 1	2 d	>95	60
6	CuI (1 eq.)	DIPEA (2 eq.)	Toluene	2 d	>95	40
7	CuI (1 eq.)	DIPEA (2 eq.)	MeOH	2 d	>95	50
8	CuBr (0.2 eq.)	DBU (3 eq.)	Toluene	>4 d	—	—
9	Cu/C (0.05 eq.)	TEA (1.5 eq.)	Dioxane	>4 d	—	—

[a] Estimated from LC-MS profiles. See ESI.

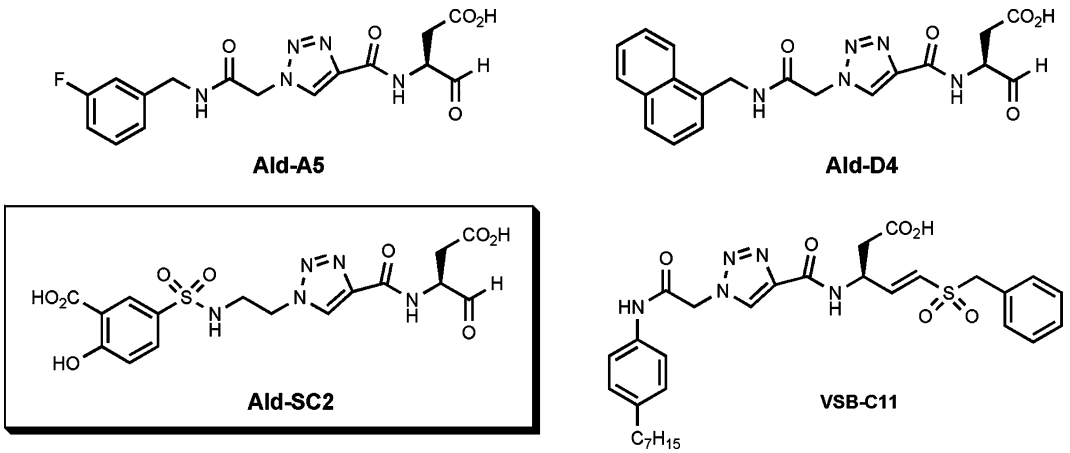


Fig. 2 Structures of initial potent hits.

to assemble all the inhibitors in a matter of 1–2 days. LC-MS characterizations of the inhibitors showed that, in almost all cases, the limiting alkyne warheads were completely consumed and the desired triazole products were quantitatively formed.[12] Thus, we concluded that the "clicked" caspase library was sufficiently pure and may be used directly for subsequent biological screenings. The inhibitory potency of the 198-member library against caspase-3 and -7 was determined using a standard fluorescence-based microplate assay. First, the so-called inhibitor fingerprint of the enzymes against the panel of inhibitors was obtained (see Fig. S5 in ESI), from which selected "hits" were identified and followed up by quantitative determination of their IC_{50} against the enzymes (Fig. 2 and Table 2). Significantly, the most potent reversible inhibitor, Ald-SC2, showed modest IC_{50} values of 4.67 μM and 7.7 μM against caspase-3 and -7, respectively. The most potent irreversible VS inhibitor, VSB-C11, on the other hand, showed an IC_{50} of 5.0 μM against caspase-7.

A number of interesting observations arose from our screening results. First, the aldehyde-containing inhibitors were in general more potent than the VS inhibitors (see ESI). This is expected, as aldehydes are amongst the most potent inhibitors known against cysteine proteases. Second, the most potent reversible inhibitor identified, Ald-SC2, contains a salicylic acid sulfonamide at the P_4 position, which coincides with the best inhibitor previously discovered by Choong and co-workers from the "extended tethering" approach.[4] This again validates the use of click chemistry as a valuable tool in drug discovery, and the triazole linker as a potential "druggable" linker. Finally, the most potent (irreversible) inhibitor identified from the VS library was VSB-C11, which has a substituted aromatic amide, rather than salicylic acid sulfonamide (as in the case of Ald-SC2) at the P_4 position. This underlines one of the main challenges facing current drug discovery—subtle changes

Table 2 IC_{50}s of selected "hits" from fingerprint experiments

		IC_{50}/μM	
Inhibitor ID	Inhibitor	Caspase-3	Caspase-7
137	Ald-A5	23.0	—
172	Ald-D4	12.7	—
197	Ald-SC2	4.67	7.7
035	VSB-C11	—	5.0

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in an inhibitor scaffold (from an aldehyde to a VS warhead in our case) may impart unpredictable effects on the overall inhibitor potency. To better understand how the inhibitors interact with the enzymes, Ald-SC2 and VSB-C11 were docked against caspase-3 and caspase-7 active sites, respectively, using the Sybyl software (see ESI). Besides the expected interactions of the salicylic acid group in Ald-SC2 with residues in the S_4 pocket, nitrogen atoms in the triazole linker were also shown to form hydrogen bonds with Ser205 located within the active site of caspase-3. In the case of VSB-C11, it was found that the long aliphatic chain in the inhibitor fits nicely into the narrow S_4 subsite of caspase-7.

In conclusion, we have developed an efficient strategy for the facile assembly of reversible (aldehyde) and irreversible (vinyl sulfone) caspase inhibitors using "click chemistry". From the hits identified, we have shown that the triazole heterocycle is indeed a suitable neutral alternative to the P_2-P_3 residues, thus making this strategy a good fragment-based approach for the high-throughput synthesis of caspase inhibitors having diverse P_4 groups. We anticipate this method will be useful to develop inhibitors against other cysteine proteases as well. Our present approach thus provides a useful chemical tool in the emerging field of "catalomics".[13]

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