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Bridged α-helix mimetic small molecules†

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Herein, we report a strategy for generating conformationally restricted α-helix mimetic small molecules by introducing covalent bridges that limit rotation about the central axis of α-helix mimetics. We demonstrate that the bridged α-helix mimetics have enhanced binding affinity and specificity to the target protein due to the restricted conformation as well as extra interaction of the bridge with the protein surface.

α-Helices represent the most common protein secondary structures and play a pivotal role as recognition motifs in many therapeutically relevant protein–protein interaction (PPI) interfaces. Therefore, the disruption of aberrant PPIs implicated in a wide range of disease states using α-helix mimetic molecules has been emerging as an attractive therapeutic strategy.[1–5] One such approach is the introduction of covalent bridges into α-helical peptides that can keep the peptides in stabilized helical conformations, leading to enhanced binding affinity as well as improved cell permeability and proteolytic stability.[6–10] As an alternative to these peptide-based approaches, Hamilton and co-workers have pioneered a small-molecule-based strategy.[11,12] They developed small-molecule α-helix mimetics in which non-peptidic small-molecule scaffolds are substituted with appropriate side chains to recapitulate the spatial orientation of critical side chains at *i*, *i* + 3 or *i* + 4, and *i* + 7 positions on α-helical peptides (Fig. 1A). The conformation of substituted side chains that match the spatial orientation is essential for binding to proteins.[13–15] A number of efforts have been devoted to develop such small-molecules,[16–31] but many of the current α-helix mimetics have unfavourable issues such as low aqueous solubility, synthetic difficulty, structural flexibility, *etc.*

In the search for novel α-helix mimetic small molecules, we recently developed α-helix mimetics based on a triazine–piperazine–triazine structure.[26] This scaffold has three functional groups (R_1, R_2, and R_3) that can match the spatial arrangement of critical side chain residues at *i*, *i* + 3 or *i* + 4, and *i* + 7 positions located on α-helices (Fig. 1B), enabling it to mimic α-helical structures and to act as an inhibitor of α-helix-mediated PPIs. We synthesized a combinatorial one-bead one-compound library of ~1500 triazine–piperazine–triazine compounds substituted with various side chains on R_1, R_2, and R_3. Affinity-based on-bead screening of this library against Mcl-1, an anti-apoptotic Bcl-2 family protein, identified **9c** as an inhibitor of the interaction between the Mcl-1 and BH3 protein family such as BAK and BAX. The compound was shown to induce apoptotic

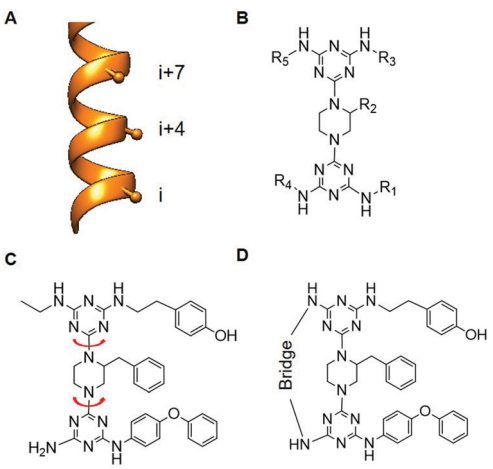

Fig. 1 Design of bridged α-helix mimetic small-molecules. (A) Structure of an α-helical peptide with *i*, *i* + 4, and *i* + 7 side-chain positions, (B) triazine–piperazine–triazine-based α-helix mimetics, (C) structure of **9c**, an Mcl-1 inhibitor, having two rotatable bonds, and (D) bridged α-helix mimetics.

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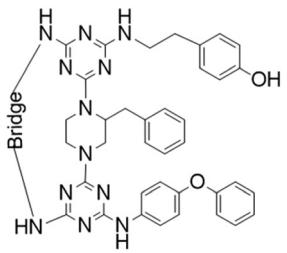
cell death in cancers by disrupting the Mcl-1/BH3 interaction. Although we have demonstrated that the designed small molecules not only have excellent aqueous solubility and synthetic accessibility but also are able to serve as α-helix mimetics, their binding affinity was somewhat weak (K_i = 9.3 μM). The triazine–piperazine–triazine scaffold can adopt numerous conformations due to the presence of two rotatable bonds between piperazine and two triazine rings (Fig. 1C). This inherent conformational flexibility may cause major entropy loss upon binding of the scaffold to proteins, thereby resulting in low binding affinity to its target. Here we report a strategy for generating conformationally restricted α-helix mimetic small molecules by limiting rotation about the central axis of α-helix mimetics.

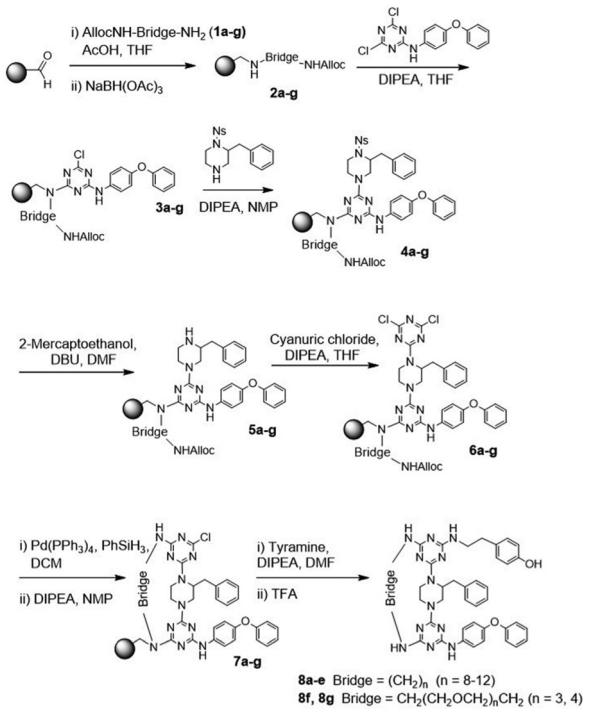
Inspired by previous studies on the conformational restriction of α-helix mimetics,[7,10,32–34] we hypothesized that if the rotational freedom of the triazine–piperazine–triazine backbone is restricted by incorporating a bridge (Fig. 1D), the binding activity of the scaffold could be improved. To examine this, we designed a conformationally restricted triazine–piperazine–triazine structure, in which the R_4 and R_5 positions of **9c** are connected by various bridges, such as hydrocarbon, polyethyleneglycol (PEG), aromatic ring- or triazole-containing, and amide-containing linkers (Table 1). In our previous work, a docking model of **9c** bound by Mcl-1 suggested that three side chain residues of **9c** occupied the BH3 binding pocket of Mcl-1, whereas R_4 and R_5 positions are exposed outside without direct interaction with Mcl-1.[26] Thus, the introduction of a bridge at these solvent-exposed sites may not affect the interaction of **9c** to Mcl-1 protein. Furthermore, appropriate bridges could restrict the rotation about the central axis of **9c**'s triazine–piperazine–triazine scaffold and thus increase its binding affinity.

Table 1 Chemical structures of designed compounds



8a-m

Compd	Chemical structure of bridge	Compd	Chemical structure of bridge
8a–e	n=6, **8a** n=7, **8b** n=8, **8c** n=9, **8d** n=10, **8e**	**8h**	
		8i	
		8j	
		8k	
8f–g	n=3, **8f** n=4, **8g**	**8l**	
		8m	



Scheme 1 Solid-phase synthesis of bridged triazine–piperazine–triazine structures (**8a–g**).

For the synthesis of the designed molecules, we developed a convenient solid-phase synthetic route (Scheme 1). Initially, the mono-Alloc-protected amines **1a–g** were loaded on PAL aldehyde resin by the reductive amination reaction, providing secondary amines **2a–g**. Next, mono-substituted dichlorotriazine was coupled to the resin-bound amines **2a–g**, and the chloride on **3a–g** was replaced with the nosyl-protected piperazine derivative. After deprotection of the nosyl group, cyanuric chloride was coupled to the resin-bound piperazines **5a–g**. Following the removal of the Alloc group on **6a–g**, on-resin cyclization using DIPEA gave bridged triazine–piperazine–triazine structures **7a–g**. A subsequent amination reaction with tyramine followed by the cleavage reaction with trifluoroacetic acid (TFA) afforded the final products **8a–g**. The cleaved products were purified by reverse-phase HPLC. For the preparation of the compounds with different bridges, the synthetic scheme was slightly modified (for more details, see Scheme S1 and Table S1, ESI†).

To examine whether the introduction of covalent bridges can lead to increased conformational rigidity of α-helix mimetics, thereby improving their binding affinity, we employed a competitive fluorescence polarization (FP) assay using purified recombinant Mcl-1 protein and a fluorescently labeled BH3 peptide (KALETL RRVGDGVQRNHETAF).[35] Among the tested compounds, **8f** and **8g** possessing PEG bridges displayed more than 6-fold improvement in their binding affinities (K_i = 2.6 μM and 2.9 μM, respectively) compared to the parent compound **9c** (Fig. 2). The enhanced binding activity of the PEG bridged compounds

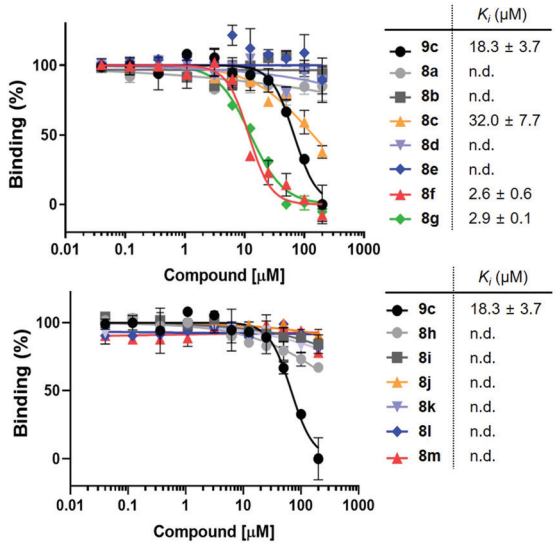


Fig. 2 Inhibition curves of **8a–m** and **9c** for fluorescein-labeled BH3 peptide binding to recombinant Mcl-1 (amino acids 172–320) as determined by competitive FP assays. Error bars represent standard deviation from three independent experiments.

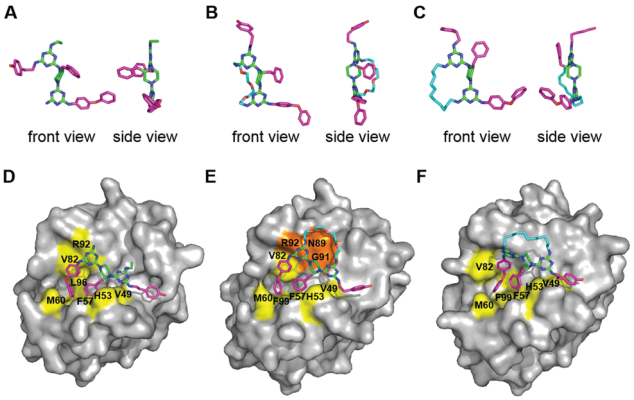


Fig. 3 Simulated structures. (A) **9c**, (B) PEG-bridged compound **8f** (red coloured in the PEG bridge is oxygen), (C) hydrocarbon-bridged compound **8d**, (D) **9c**/Mcl-1 complex, (E) **8f**/Mcl-1 complex, and (F) **8d**/Mcl-1 complex. Yellow and orange colours indicate the interaction between the molecules and Mcl-1.

(**8f** and **8g**) might be attributed to the restricted rotation of the triazine–piperazine–triazine axis. In contrast, the compounds bridged by various hydrocarbon linkers showed even decreased or no binding affinity. For compounds **8a–c** containing relatively short bridges, ring strain might bend the triazine–piperazine–triazine structure, causing changes in the spatial location of three side chains and thereby deteriorating the binding affinity. To our surprise, however, compounds **8d–e** were also found to have no binding affinity although their bridges have the same or similar length to that of PEG bridged compound **8f**. One explanation that might account for this observation is that hydrocarbon bridges on **8d–e** may interfere with their binding to the target protein. The hydrophobicity of all-hydrocarbon bridges may be unfavourable for protein binding due to the hydrophilic nature of the protein surface. Likewise, compounds **8h–m** with the other bridges including aromatic ring or triazole-containing bridges did not exhibit improved binding affinities presumably due to the hydrophobic nature of their bridges (Fig. 2). Indeed, stabilization of helical peptides by all-hydrocarbon bridges often compromises the binding affinities in spite of their increased helical propensity.[10,36,37]

To investigate the impact of covalent bridges and their chemical nature on the binding activity, we conducted computational studies on the parent (**9c**), PEG-bridged (**8f**), and hydrocarbon-bridged (**8d**) compounds (Fig. 3). We first performed flexible docking simulations of these compounds to the Mcl-1 surface and then carried out fully atomistic, explicit-water molecular dynamics simulations to examine the stability of the docked complexes (ESI†). We found that the binding positions of these compounds are similar to that of the BH3 protein (the surface region coloured in yellow in Fig. 3D–F; see also Fig. S4, ESI†). However, a contrasting behavior was observed for the bridge parts depending on their chemical nature; while the PEG bridge in **8f** forms additional electrostatic interactions with the Mcl-1 surface (the region coloured in orange in Fig. 3E), the hydrocarbon bridge in **8d** does not provide such additional contacts (Fig. 3F). This may be because the purely hydrophobic bridge parts are located close to the hydrophilic surface residues (N81, G83, and R84) of Mcl-1.

Based on these results, we selected two PEG-bridged compounds (**8f** and **8g**) as the best inhibitors for further studies. Before starting cellular assays, we evaluated their aqueous solubility by calculating the partition coefficient. As shown in Table S2 (ESI†), the $c\log P$ values of **8f** and **8g** were 2.38 and 2.05, respectively, suggesting that they have a desirable drug-like property in terms of their hydrophilicity. We then measured their solubility in water. In good agreement with the $c\log P$ prediction, both compounds exhibited good solubility in water (>100 μg mL^{-1}), which is above the desirable aqueous solubility for favourable oral biopharmaceutical properties.[38]

To assess the ability of the compounds to inhibit the Mcl-1/BAK interaction in living cells, we performed coimmunoprecipitation experiments. Jurkat T lymphocyte cells were treated with DMSO or increasing concentrations of **8f** or **8g**. The effect of **8f** and **8g** on the Mcl-1/BAK interaction in cells was analysed by Mcl-1 immunoprecipitation and BAK Western blot (Fig. 4A and Fig. S6, ESI†). In the cells treated with **8f** or **8g**, immunoprecipitated BAK protein was significantly decreased in a dose-dependent manner. This clearly demonstrates that **8f** and **8g** are not only cell-permeable, but also able to disrupt the Mcl-1 interaction with BAK in cells.

Because Mcl-1 blocks apoptosis by sequestering the pro-apoptotic proteins such as BAK, disrupting the Mcl-1/BH3 interaction by its inhibitors would lead to apoptosis in cancer cells. To test this, Jurkat T lymphocyte cells were treated with varying concentrations of **8f**, **8g**, or **9c**, and caspase activity was measured to monitor apoptosis (Fig. 4B). Not surprisingly, **8f** and **8g** were far more effective at triggering apoptosis than **9c**. The EC_{50} values of **8f** and **8g** are in the low micromolar ranges, which are consistent with their *in vitro* binding affinities (Fig. 4B). To validate the apoptotic effect of **8f** and **8g**, we used flow

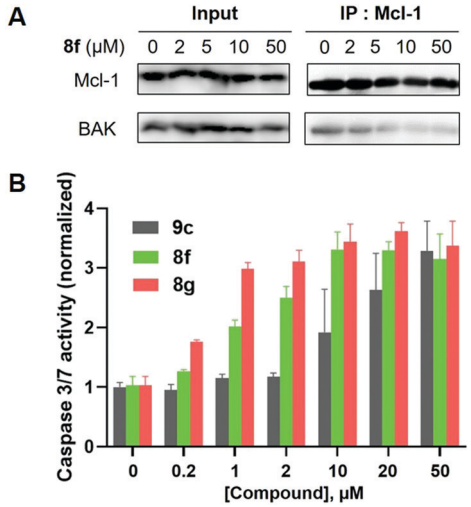


Fig. 4 Cellular activities of **8f** and **8g** on Jurkat T lymphocyte cells. (A) Co-immunoprecipitation experiments with **8f**. The results are representative of three independent experiments. (B) Caspase 3/7 activity assays. Error bars represent standard deviations from three independent experiments.

cytometry. As seen in Fig. S7 (ESI†), **8f** and **8g** exhibited a dose-dependent increase of cells undergoing apoptosis but not necrosis whereas the treatment of **9c** resulted in increases of both apoptotic and necrotic cells, indicating the specificity of **8f** and **8g**. To further assess the selectivity of **8f** and **8g** for Mcl-1, we tested whether these compounds could inhibit the other PPIs that are also mediated by α-helices, such as Bcl-xL/BH3 and NCOA1/STAT6 interactions (Fig. S8, ESI†). Although these proteins have similar binding pockets to accommodate α-helical peptides, **8f** and **8g** had no inhibitory effect on both PPIs, demonstrating the selectivity of both compounds for Mcl-1 over the other proteins.

In summary, we have developed a strategy for creating conformationally restricted α-helix mimetic small molecules by introducing a covalent bridge, thereby limiting rotation about the central axis of α-helix mimetics. We have demonstrated that the bridged α-helix mimetics have substantially enhanced binding affinity and specificity to the target protein likely due to the restricted conformation as well as extra interaction of the bridge with the protein surface. Note that our bridged α-helix mimetics exhibit excellent cell-permeability and aqueous solubility. We believe that our strategy will be a highly useful means of generating potent and selective α-helix mimetics as PPI inhibitors.

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Conflicts of interest

There are no conflicts to declare.

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