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Design, Solid-Phase Synthesis, and Evaluation of a Phenyl-Piperazine-Triazine Scaffold as α-Helix Mimetics

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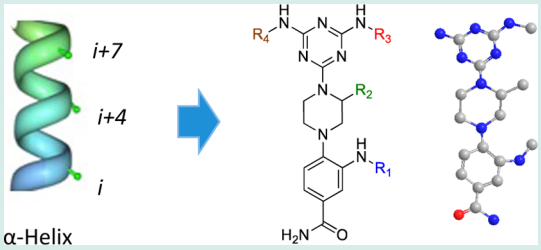
Supporting Information

ABSTRACT: α-Helices play a critical role in mediating many protein−protein interactions (PPIs) as recognition motifs. Therefore, there is a considerable interest in developing small molecules that can mimic helical peptide segments to modulate α-helix-mediated PPIs. Due to the relatively low aqueous solubility and synthetic difficulty of most current α-helix mimetic small molecules, one important goal in this area is to develop small molecules with favorable physicochemical properties and ease of synthesis. Here we designed phenyl-piperazine-triazine-based α-helix mimetics that possess improved water solubility and excellent synthetic accessibility. We developed a facile solid-phase synthetic route that allows for rapid creation of a large, diverse combinatorial library of α-helix mimetics. Further, we identified a selective inhibitor of the Mcl-1/BH3 interaction by screening a focused library of phenyl-piperazine-triazines, demonstrating that the scaffold is able to serve as functional mimetics of α-helical peptides. We believe that our phenyl-piperazine-triazine-based α-helix mimetics, along with the facile and divergent solid-phase synthetic method, have great potential as powerful tools for discovering potent inhibitors of given α-helix-mediated PPIs.



KEYWORDS: *α-helix mimetics, solid-phase synthesis, combinatorial library, protein−protein interaction inhibitor*

INTRODUCTION

Protein−protein interactions (PPIs) play a critical role in mediating a wide range of cellular functions, and their misregulation is implicated in numerous disease states. Molecules that are capable of modulating specific PPIs can be not only valuable research tools to interrogate molecular functions of target proteins, but also be potential therapeutic candidates.[1−9] However, developing such molecules is a daunting task primarily due to relatively large and flat interfaces involved in PPIs. Typical druglike small molecules may not be suitable to effectively cover such extended protein contact surfaces, and indeed a number of high-throughput screens have failed to identify PPI inhibitors. Interestingly, however, many PPIs are mediated by so-called "hot spots", compact regions in protein interfaces, where only a few amino acid residues are critical for PPIs.[1] An example is the PPIs mediated by α-helical peptides. The general characteristics of the α-helix-mediated PPIs is that short helical peptides spanning two or three helical turns act as recognition motifs where three or four side chains at *i*, *i*+3 or *i*+4, and *i*+7 amino acid positions are often involved in critical interactions (Figure 1). Since α-helix-mediated PPIs play a pivotal role in many disease states, there have been tremendous efforts to develop functional mimetics of α-helical peptide segments to modulate such interactions.[10,11]

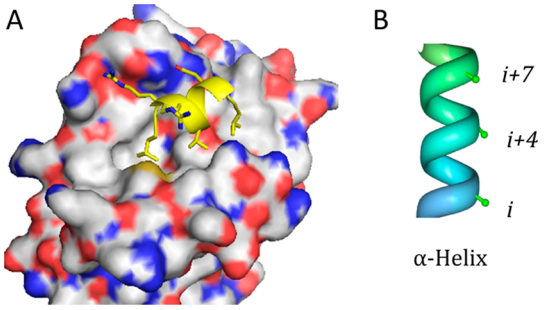


Figure 1. (A) X-ray crystal structure (PDB ID 3mk8) of Mcl-1 in complex with a BH3 peptide. In this interaction, three hydrophobic residues (Leu_{213}, Val_{216}, and Val_{220}) at the *i*, *i*+4, and *i*+7 positions on the BH3 peptide are critical for binding. (B) α-Helix with *i*, *i*+4, and *i* +7 side chain positions.

Hamilton and colleagues first demonstrated the idea that properly functionalized terphenyl and related structures are capable of mimicking short α-helical peptide structures and

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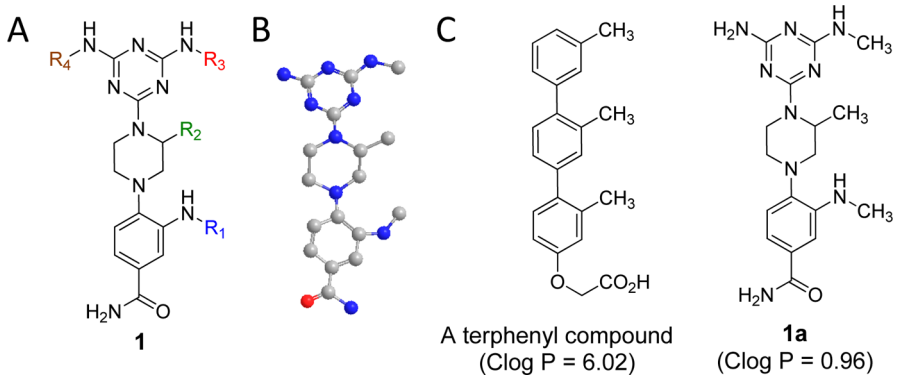
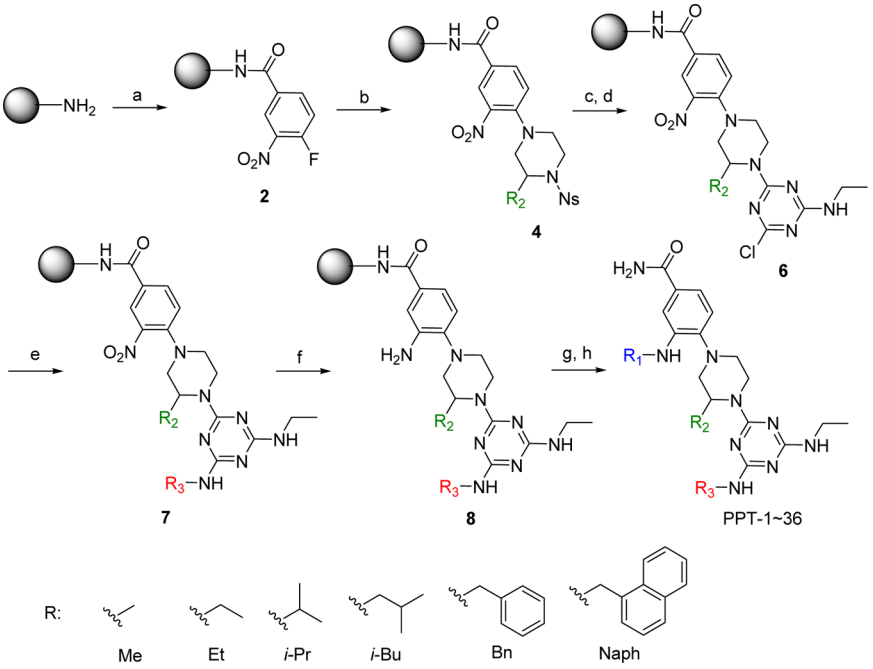


Figure 2. α-Helix with i, i+4, and i+7 side chain positions. (A) Phenyl-piperazine-triazine-based α-helix mimetic scaffold **1**. (B) An energy-minimized structure of **1a** (R_1, R_2, R_3 = Me; R_4 = H). (C) Comparison of ClogP values for a terphenyl structure and **1a**.

Scheme 1. Solid-Phase Synthesis of Phenyl-Piperazine-Triazines[a]



[a]Reagents and conditions: (a) 4-Fluoro-3-nitrobenzoic acid, HATU, DIEA, DMF, rt, 24 h; (b) *N*-Nosyl protected piperazine derivatives **3**, DIEA, DMF, 95 °C, 12 h; (c) 2-Mercaptoethanol, DBU, DMF, rt, 3 h; (d) 2-Aminoethyl-4,6-dichloro-[1,3,5]triaizine **5**, DIEA, THF, 60 °C, 3 h; (e) R_3NH_2, DIEA, NMP, 80 °C, overnight; (f) $SnCl_2{\cdot}2H_2O$, DMF, rt, 24 h, (g) Condition A (for aromatic aldehyde): R_1CHO, $CH(OMe)_3$/DMF/MeOH 9:1:2 (1% HOAc), 50 °C, 18 h; then $NaCNBH_3$, THF, 50 °C, 6 h, Condition B (for aliphatic aldehyde): R_1CHO, benzotriazole, $CH(OMe)_3$/DMF/MeOH 9:1:2 (1% HOAc), rt, 18 h; then $NaCNBH_3$, THF, rt, 6 h; (h) 95% TFA in CH_2Cl_2, rt, 3 h.

thus acting as inhibitors of α-helix-mediated PPIs.[12−16] Since the pioneering work, a number of terphenyl-inspired molecules have been developed over the past decade.[13,15−32] Although terphenyl-related scaffolds are emerging as a promising class of α-helix mimetics, there are several unfavorable issues such as low water solubility and synthetic complexity. Terphenyl-based structures inherently have high hydrophobicity, making them poorly aqueous soluble. Moreover, most terphenyl-based structures are synthesized by linear, long synthetic routes. Synthetic difficulty not only limits large library construction, but also makes it difficult to modify initial lead structures to improve their potency. Therefore, one important goal in this field is to develop small molecules with favorable physicochemical properties and ease of synthesis. Despite several approaches to the development of such molecules,[13,17,20,22,23,33] it still remains problematic. Recently, we also have reported new scaffolds as α-helix mimetics such as pyrrolopyrimidines and triazine-piperazine-triazines.[34−36] As part of our continuing efforts to address this issue, here we describe the design of an α-helix mimetic scaffold based on a phenyl-piperazine-triazine structure that possesses ease of synthetic manipulation and favorable physicochemical properties. We then identified a selective inhibitor of the Mcl-1/BH3 interaction by screening a focused library of phenyl-piperazine-triazines, demonstrating that the designed molecules can serve as α-helix mimetics.

RESULTS AND DISCUSSION

Inspired by terphenyl scaffolds, we designed phenyl-piperazine-triazine scaffold **1** (Figure 2A) as nonpeptidic, α-helix mimetic small molecules. We envisioned that the scaffold **1** with suitably functionalized R_1, R_2, and R_3 groups could serve as α-helix mimetics. Energy-minimization study predicted that the 3-

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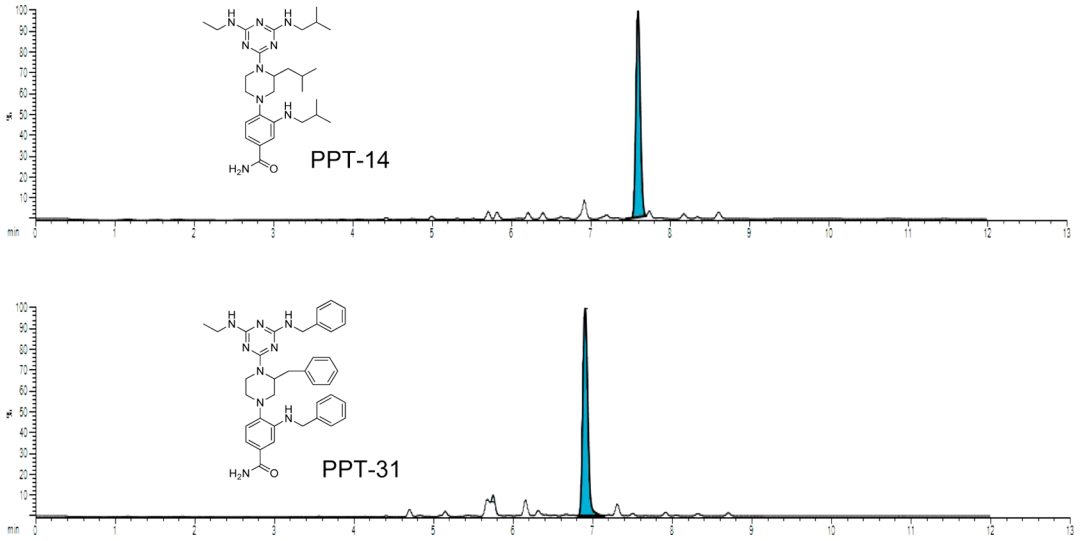
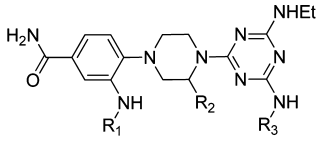


Figure 3. Representative HPLC traces of crude final products.

Table 1. Synthesized Compounds for Evaluation as α-Helix Mimetics



compound	R_1	R_2	R_3	purity[a] (%)	compound	R_1	R_2	R_3	purity[a] (%)
PPT-01	Et	*i*-Bu	*i*-Pr	94	PPT-19	*i*-Bu	Bn	Bn	93
PPT-02	Et	*i*-Bu	*i*-Bu	83	PPT-20	*i*-Bu	Bn	Naph	95
PPT-03	Et	*i*-Bu	Bn	86	PPT-21	*i*-Bu	Me	*i*-Pr	74
PPT-04	Et	*i*-Bu	Naph	85	PPT-22	*i*-Bu	Me	*i*-Bu	74
PPT-05	Et	Bn	*i*-Pr	95	PPT-23	*i*-Bu	Me	Bn	74
PPT-06	Et	Bn	*i*-Bu	87	PPT-24	*i*-Bu	Me	Naph	78
PPT-07	Et	Bn	Bn	95	PPT-25	Bn	*i*-Bu	*i*-Pr	89
PPT-08	Et	Bn	Naph	69	PPT-26	Bn	*i*-Bu	*i*-Bu	95
PPT-09	Et	Me	*i*-Pr	83	PPT-27	Bn	*i*-Bu	Bn	83
PPT-10	Et	Me	*i*-Bu	83	PPT-28	Bn	*i*-Bu	Naph	92
PPT-11	Et	Me	Bn	81	PPT-29	Bn	Bn	*i*-Pr	82
PPT-12	Et	Me	Naph	77	PPT-30	Bn	Bn	*i*-Bu	93
PPT-13	*i*-Bu	*i*-Bu	*i*-Pr	89	PPT-31	Bn	Bn	Bn	94
PPT-14	*i*-Bu	*i*-Bu	*i*-Bu	94	PPT-32	Bn	Bn	Naph	91
PPT-15	*i*-Bu	*i*-Bu	Bn	97	PPT-33	Bn	Me	*i*-Pr	70
PPT-16	*i*-Bu	*i*-Bu	Naph	88	PPT-34	Bn	Me	*i*-Bu	63
PPT-17	*i*-Bu	Bn	*i*-Pr	94	PPT-35	Bn	Me	Bn	70
PPT-18	*i*-Bu	Bn	*i*-Bu	95	PPT-36	Bn	Me	Naph	65

[a]Purity of crude final products was determined by HPLC.

dimensional arrangements of the three functional groups (R_1, R_2, and R_3) in compound **1** can effectively mimic the spatial orientation of the three critical residues (*i*, *i* + 4, and *i* + 7) of α-helices (Figure 2B). Moreover, the phenyl-piperazine-triazine scaffold **1** is expected to have more favorable physicochemical properties compared to traditional terphenyl scaffolds because of the presence of several heteroatoms and an aliphatic piperazine ring instead of an aromatic ring. To evaluate their aqueous solubility, we calculated the partition coefficients. The ClogP (calculated log $P_{octanol/water}$) values for a phenyl-piperazine-triazine compound **1a** and a terphenyl scaffold are 0.96 and 6.02, respectively (Figure 2C). This result indicates that our scaffold possesses significantly improved water solubility compared to terphenyl structures. To the best of our knowledge, the phenyl-piperazine-triazine scaffold has never been explored as α-helix mimetics, while the scaffold itself (without R_1-R_3-like side chains) has been previously tested as allosteric modulators of the follicle stimulating hormone receptor.[37]

Combinatorial synthesis by solid-phase chemistry is a widely used method for the rapid preparation of diversified compound collections. In contrast, most terphenyl-like scaffolds are prepared by solution-phase synthesis, and there are only a few reports on solid-phase synthesis of nonpeptidic, small molecule α-helix mimetics.[25,33−35,38] While solution-phase synthesis is useful in some cases (e.g., Boger's method for a 8000-member library of α-helix mimetics),[39] solid-phase synthesis has proven to be a powerful tool for large library

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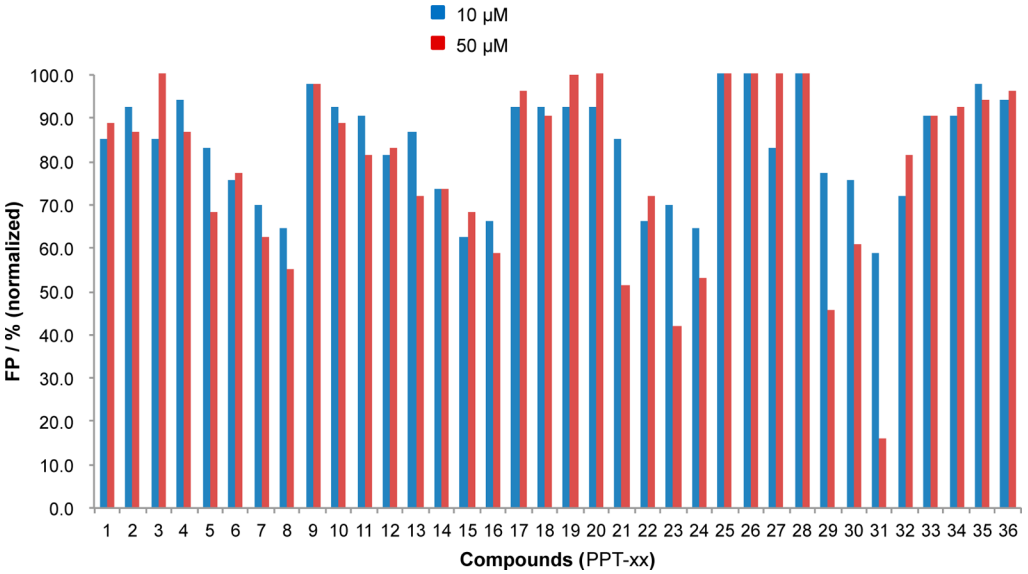

Figure 4. Inhibitory effect of phenyl-piperazine-triazines on the interaction between Mcl-$1_{172-320}$ and a fluorescein-labeled BH3 peptide. Fluorescence polarization was measured using 36 compounds at 10 μM (blue bars) and 50 μM (red bars).

construction, as well as further structural optimization of initial lead compounds. We developed a facile solid-phase synthetic route to prepare the phenyl-piperazine-triazine scaffold **1** (Scheme 1). It is noteworthy that while a solution-phase synthesis of this scaffold has been reported,[37] here we describe for the first time a solid-phase method for the synthesis of the phenyl-phenyl-piperazine-triazine scaffold.

First, 4-fluoro-3-nitrobenzoic acid was loaded on Rink-Amide MBHA resin using standard peptide coupling conditions.[40] The resulting compound **2** was reacted with 2-nitrobenzenesulfonyl (Ns)-protected piperazine derivatives **3** to give **4**. The *N*-Ns protecting group on **4** was then removed by treating with 2-mercaptoethanol. Subsequently, 2-ethylamino-4,6-dichloro-[1,3,5]triazine **5** was coupled to the resin-bound piperazine to produce **6**. The chloride on the triazine in compound **6** was subsequently displaced with different amines (R_3NH_2). Next, the nitro group on **7** was reduced by reacting with $SnCl_2$ in DMF. The resultant amines **8** were alkylated by reductive amination reactions with various aldehydes to introduce R_1 functional groups. While the reaction with aromatic aldehydes gave the expected alkylated products in high yield, the reductive alkylation with aliphatic aldehydes under the same reaction conditions provided dialkylated products as major products. To avoid these byproducts by overalkylation, benzotriazole was added to the reaction mixture. Benzotriazole is known to inhibit the formation of dialkylated byproducts in reductive amination.[40] Indeed, no dialkylation was observed in the presence of benzotriazole. Finally, the trisubstituted phenyl-piperazine-triazines were cleaved from the resin by treating with 95% trifluoroacetic acid (TFA). After cleavage reaction, the identity and purity of the crude products were determined by LC/MS analysis (Figure 3 and Table 1). The average purity of the crude final products was >85%, illustrating the robustness of our solid-phase synthetic route. Importantly, since our solid-phase synthetic method is facile and divergent, it is amenable to rapid construction of a large combinatorial library of structurally diverse α-helix mimetics and synthesis of derivatives by using readily available monomer building blocks. Notably, piperazine derivatives with various side chains are not only commercially available, but also can be efficiently synthesized.[23,27,41]

Next, we examined whether the designed phenyl-piperazine-triazine scaffold is capable of mimicking α-helical peptide segments. To test this, we selected the Mcl-1/BH3 interaction as the target. This interaction is critically dependent on a short α-helical peptide segment in which three key residues from BH3 proteins bound to a hydrophobic cleft on Mcl-1 are essentially important for the interaction (Figure 1A).[42] By its binding to the pro-apoptotic BH3 proteins such as BAK, Mcl-1 neutralizes their pro-apoptosis functions and enhances cell survival. Therefore, development of potent inhibitors of the Mcl-1/BH3 interaction is of great interest as a promising anticancer strategy. It is of note that selective Mcl-1 inhibitors are rare.[42−44]

Using the synthetic procedure described in Scheme 1, we constructed a small focused library of the phenyl-piperazine-triazines (Table 1). We selected various hydrophobic side chain residues for R_1, R_2, and R_3 to mimic three hydrophobic residues (Leu, Val, and Val) of the BH3 helical peptide (Table 1). The 36 library molecules (PPT-01−PPT-36) were screened for their ability to disrupt the interaction between Mcl-$1_{172-320}$ (a deletion construct containing the BH3-binding groove) and a known fluorescently labeled BH3 peptide in two different concentrations (10 μM and 50 μM). The screening results are depicted in Figure 4. From this competitive fluorescence polarization assay, PPT-31 was chosen as the best candidate as it showed not only the most potent inhibitory activity at both 10 and 50 μM, but also good concentration dependence. The hit compound, PPT-31, contains three phenyl groups at R_1, R_2, and R_3 (Figure 5A). On the basis of molecular modeling, these three phenyl groups on PPT-31 are predicted to overlay with the three key residues Val_{220}, Val_{216} and Leu_{213} on the α-helical BH3 peptide bound to a hydrophobic cleft on Mcl-1 (Figure 5B). The binding affinity of PPT-31 was determined using the same competitive FP assay (K_i = 7.3 μM) (Figure 5C). We then examined whether this compound has the selectivity, because the BH3 binding pocket of Mcl-1 is similar to those of other Bcl-2 family proteins such as Bcl-xL. We tested the binding affinity of PPT-31 to Bcl-xL. As shown in Figure 5C, PPT-31 did not disrupt the interactions between Bcl-xL_{1-212} (a deletion construct containing the BH3-binding pocket) and

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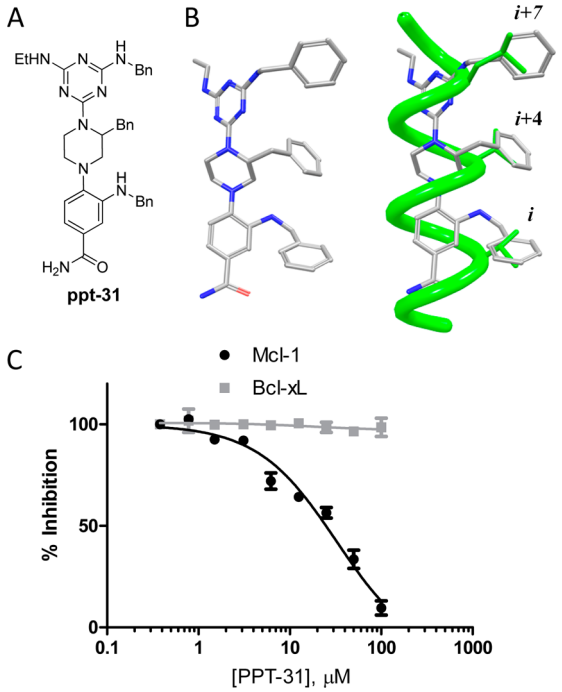

Figure 5. (A) Chemical structure of hit compound, PPT-31. (B) An energy-minimized structure of PPT-31 and overlay with an α-helical peptide. (C) Inhibition curves of PPT-31 for a fluorescein-labeled BH3 peptide binding to Mcl-$1_{172-320}$ (black) and Bcl-xL_{1-212} (gray). Error bars represent standard deviation from three independent experiments.

BH3, suggesting it can be a selective Mcl-1 inhibitor. Further biological studies are underway. Together, we demonstrated that a phenyl-piperazine-triazine scaffold with appropriately functionalized side chains could act as an inhibitor of α-helix mediated PPIs.

In summary, we have designed a phenyl-piperazine-triazine-based α-helix mimetics having improved water solubility and excellent synthetic accessibility. We also developed a simple and divergent solid-phase synthetic route that allows for the creation of a large, diverse library of α-helix mimetics. Further we identified a selective inhibitor of the Mcl-1/BH3 interaction from a preliminary set of 36 phenyl-piperazine-triazines, demonstrating that our scaffold is capable of acting as functional mimetics of α-helical peptides. With the promising results described here, we are currently constructing a much larger combinatorial library of phenyl-piperazine-triazines by employing structurally diverse monomer building blocks at the four positions (R_1, R_2, R_3, and R_4) (Figure 1A). Consequently, we believe that our phenyl-piperazine-triazine-based α-helix mimetics, along with the convenient solid-phase synthetic method, hold great potential as powerful tools for discovering potent inhibitors of given α-helix-mediated PPIs.

EXPERIMENTAL PROCEDURES

General Procedure for the Synthesis of Phenyl-Piperazine-Triazines. Rink amide MBHA resin (100 mg, 93 μmol) was swelled in DMF (2 mL) for 1 h. The Fmoc group was removed by treating with 20% piperidine in DMF (2 × 10 min). The resin was thoroughly washed with DMF (3×), MeOH (2×), CH_2Cl_2 (2×), and DMF (3×) at the end of each reaction step unless otherwise noted. To the resin was added a solution of 4-fluoro-3-nitrobenzoic acid (70 mg, 378 μmol), HATU (142 mg, 378 μmol), and DIEA (130 μL, 756 μmol) in DMF (1.5 mL), and the mixture was agitated for 12 h at room temperature. Nosyl-protected piperazines **3** (4 equiv) were then coupled to resin-bound 4-fluoro-3-nitrobenzamides **2** using DIEA (10 equiv) in DMF at 95 °C for 12 h. The *N*-Nosyl group on **4** was deprotected by treating with 2-mercaptoethanol (20 equiv) and DBU (10 equiv) in DMF for 3 h at room temperature. Next, 2-ethylamino-4,6-dichloro-[1,3,5]triazine **5** (10 equiv) was introduced using DIEA (5 equiv) in THF at 60 °C for 3 h, yielding phenyl-piperazine-triazines **6**. The chloride on **6** was then displaced with various amines (R_3NH_2, 5 equiv) in the presence of DIEA (10 equiv) in NMP at 80 °C overnight to afford **7**. A solution of $SnCl_2 \cdot 2H_2O$ (60 equiv) in DMF was added to the resin, and the mixture was shaken at room temperature for 24 h. Resin-bound amine **8** was treated with a 0.4 M solution of the aldehydes (10 equiv) in dry $CH(OMe)_3$/DMF = 9:1 and a 20% (v/v) solution of acetic acid in methanol at 50 °C for 18 h. Subsequently, a 5 M solution of $NaCNBH_3$ (50 equiv) in THF was added, and the mixture was agitated at 50 °C for another 6 h to afford alkylated products. In case using aliphatic aldehydes, benzotriazole (100 equiv) was added to avoid dialkylated byproducts. Finally, cleavage reaction with TFA furnished trifunctionalized phenyl-piperazine-triazines. The identity and purity of the crude products were confirmed by LC/MS analysis. The compounds were purified by reverse-phase HPLC, and further characterized by ^{1}H NMR, ^{13}C NMR, and HRMS.

Protein Purification. The plasmids expressing BH3-binding domain of human Mcl-$1_{172-320}$ (amino acids 172−320) or human Bcl-xL_{1-212} (amino acids 1−212) tagged with GST were transformed into BL21 (DE3) *Escherichia coli* bacterial cells, and protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cell pellets were resuspended in lysis buffer (20 mM Tris, pH 7.2, 250 mM NaCl, complete protease tablet (Roche)) and lysed by sonication. After centrifugation, cell lysates were applied to a 5 mL GSTrap HP column (GE Life Sciences) according to the manufacturer's instructions. Mcl-$1_{172-320}$ and Bcl-xL_{1-212} were obtained by cleavage reaction by thrombin protease, another round of loading to GSTrap HP column to remove GST, and passing through the size exclusion chromatography.

Fluorescence Polarization Assays. For competitive fluorescence polarization experiments, we monitored the displacement of a fluorescently labeled Bak-BH3 peptide from Mcl-$1_{172-320}$ by inhibitors as described previously. Briefly, 10 nM of TAMRA-labeled Bak-BH3 (TAMRA-Abu-KAL-ETLRRVGDGVQRNHETAF-NH2) peptide was incubated with 0.8 μM of Mcl-$1_{172-320}$, in binding buffer (50 mM Tris, 100 mM NaCl, 20 nM of bovine serum albumin, pH 8.0) of a final volume of 60 μL in black Costar 384-well plates at room temperature for 30 min in the dark. Varying concentrations of phenyl-piperazine-triazines in 40 μL of binding buffer were added to the mixture. After incubation for 15 min at room temperature, the fluorescence polarization values (mP units) were measured by an Infinite 200 PRO Microplate Reader (Tecan). Excitation wavelength was 485 nm, and emission was detected at 535 nm. K_i values were calculated as described previously.[45]

ASSOCIATED CONTENT

Supporting Information

Materials and general methods, characterization of final products, LC-MS traces of purified products, and ^{1}H and ^{13}C

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NMR spectral data of representative compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

H.M. and W.S.L. contributed equally.

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Notes

The authors declare no competing financial interest.

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