Peptide directed binding; a novel approach for the discovery of modulators of alpha-helix mediated protein-protein interactions demonstrated with apoptosis regulating Mcl-1

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Abstract: *Targeting PPIs with small molecules can be challenging due to large, hydrophobic binding surfaces. Here, we describe a strategy that exploits selective alpha-helical PPIs, transferring these characteristics to small molecules. The proof-of-concept is exemplified with the apoptosis regulator Mcl-1, commonly exploited by cancers to avoid cell death. Peptide directed binding uses few synthetic transformations, requires the production of a small number of compounds and generates a high percentage of hits. In this example ~50% of the small molecules prepared showed an IC_{50} less than 100 µM and ~25% had IC_{50} values less than 1 µM to Mcl-1. Compounds show selectivity for Mcl-1 over other anti-apoptotic proteins, possess cytotoxicity to cancer cell lines, and induce hallmarks of apoptosis. This approach represents a novel and economic process for the rapid discovery of new alpha-helical PPI modulators.*

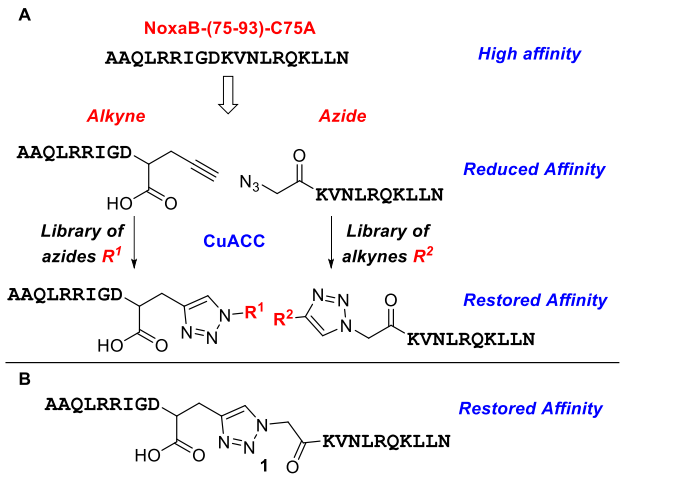
Protein-protein interactions (PPIs) regulate many processes in life, both in healthy and disease states[1] and almost two-thirds of protein-protein interfaces have alpha-helical binding motifs.[2] However, targeting PPIs can be difficult due to their large hydrophobic binding surfaces.[3] There are currently three commonly employed approaches to develop modulators of PPIs;[4] fragment screening,[5] computational screening and drug design,[6] and the exploration of peptides and peptidomimetics.[7] However, computational design and fragment screening require large libraries of molecules and extensive synthetic work, often resulting in non-selective compounds.[3] Peptides are challenging drug leads because *in vivo* their efficacy can be compromised due to a loss of secondary structure, poor cellular uptake and susceptibility to proteolysis.[8]

The work described here exploits the advantages of the above approaches while limiting their weaknesses. This approach, termed peptide directed binding, utilises the tight and selective binding of alpha-helical peptides which govern PPIs as a framework for the discovery of small molecules. Sections of the natural peptide are employed to identify a small molecule fragment which emulates the peptide.

Inspiration for this approach was taken from the REPLACE strategy of McInnes *et al.*[9] and the chimeric inhibitors of the 14-3-3/Tau PPI of Ottmann *et al.*[10] The work also exploits the advantages of altering peptidic binders, demonstrated by Gellman[11], Fairlie[12] and Wilson.[13] The technique demonstrated here improves these strategies, substituting up to ten amino acids with one small molecule fragment, and rapidly supplanting the entire peptide chain with a small molecule modulator.

In this proof-of-concept study the alpha-helical PPI of Mcl-1 and Noxa, members of the apoptosis regulating Bcl-2 family of proteins, was employed as an example.[14] The anti-apoptotic proteins (e.g Bcl-2, Bcl-x_L and Mcl-1) are often overexpressed in cancer contributing to the development of the tumour and resistance to current therapies.[15]

Noxa displays high selectivity towards Mcl-1.[16] The NoxaB (NoxaB-(75-93)-C75A) peptide is 19 amino acids (AAQLRRIGDKVNLRQKLLN) from the BH3 binding region of Noxa, and has been shown to bind tightly as an alpha-helix in the binding groove of Mcl-1.[17] NoxaB was divided into amino acids 75-84 (AAQLRRIGD) and 85-93 (KVNLRQKLLN), each possessing two key binding residues (L78, I81, V85 and Q89[17]) and reactive terminals were attached (Scheme 1 A). These peptides possessing reactive terminals displayed no appreciable binding affinity for Mcl-1 (IC_{50} values >100 µM in a previously reported competitive fluorescence anisotropy (FA) assay[16c]) in-line with results reported by Colman *et al.*[17] These reactive terminals were then utilised to perform copper catalyzed azide-alkyne cycloaddition (CuACC) reactions attaching small molecule fragments (R^1 and R^2).[18] If binding affinity is restored for these small molecule-peptide hybrids (AAQLRRIGD-R^1 and R^2-KVNLRQKLLN), then the attached small molecule fragment may represent a good emulator for that particular section of NoxaB.



Scheme 1. A: Conceptual diagram of peptide directed binding to target PPIs. B: Extended NoxaB peptide fragment which demonstrated a restored affinity for Mcl-1

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Supporting Information Statement

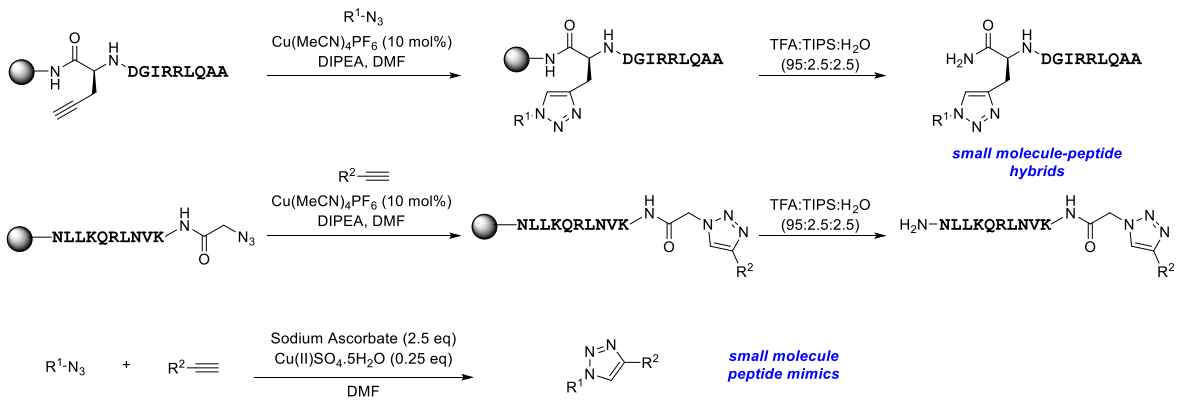
As an initial control for this strategy, the two peptide fragments were clicked together to generate an extended NoxaB peptide which had an IC_{50} of 7.23 ± 0.88 µM, compared to 650 ± 130 nM for NoxaB, highlighting the suitability of the approach (Scheme 1, B).

To economise peptide directed binding, covalent docking studies using the Schrodinger Suite were employed to assist in the identification of the small molecule fragments most likely to mimic a section of the NoxaB peptide. The crystal structure geometry of the NoxaB peptide with Mcl-1 (PDB Entry: 2NLA[17]) was modified; virtually, amino acids 85-93 of NoxaB were removed and propargylglycine was attached to the *C*-terminus of amino acids 75-84. Covalent docking studies were performed on a catalogue of azides, modelling a Huisgen cycloaddition (detailed in SI). The results were scored and ranked,[19] and those highly ranked structures which were synthetically and economically viable were chosen for synthesis. Similarly, amino acids 75-84 of the peptide were virtually removed and azidoacetic acid was attached to the *N*-terminus of amino acids 85-93.

In this manner, we selected sixty hybrid compounds, to synthesise and screen for the ability to disrupt the Mcl-1/Noxa PPI. Using solid phase peptide synthesis (SPPS) amino acids 85-93 (KVNLRQKLLN) of NoxaB were prepared and azidoacetic acid was used to cap the peptide, (Scheme 2). Subsequently alkynes were exposed to the peptide on the resin in the presence of $Cu(MeCN)_4PF_6$ and DIPEA in DMF to achieve CuAAC. The use of a N-coordinated Cu(I) source was found to be advantageous, as other Cu(I) sources gave lower yields, presumably due to sequestration of the copper catalyst by peptidic co-ordination. Cleavage from the resin and RP-HPLC provided a library of thirty-five R_2-KVNLRQKLLN hybrids. The analogous methodology was applied to prepare twenty-five AAQLRRIGD-R_1 hybrids, via a propargylglycine-terminated SPPS resin (Scheme 2).

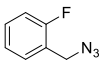
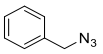
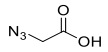
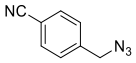
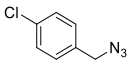
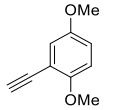
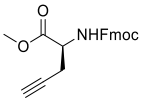
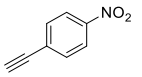
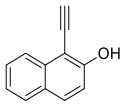
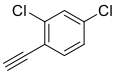
The ability of these 60 hybrids to inhibit the interaction of Mcl-1 and FITC-Noxa was examined in a FA assay, 23 compounds (30%) were identified as hits (defined as having an IC_{50} <100 µM). Eight of the hits contained amino acids 75-84, and 13 hits were derived from amino acids 85-93 (Table 1 and SI Table 1).

The orthogonal nature of the reaction enabled the combination of the azide and alkyne small molecule fragments to generate a library of small molecule-triazoles which in theory have an increased likelihood of possessing characteristics that emulate the entire NoxaB peptide. The identified hybrid molecules suggest 104 triazoles for preparation. We selected thirty-five for synthesis and evaluated them in the FA assay (Scheme 2). Nineteen (54%) of the triazole compounds showed an IC_{50} <100 µM, and ten (27%) of the compounds displayed an IC_{50} <1 µM (Table 2 and SI Table 2).



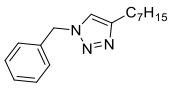
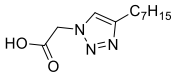
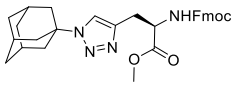
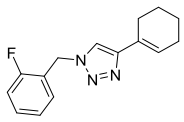
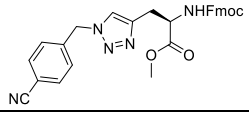
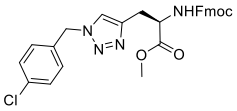
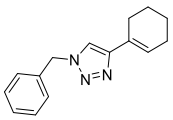
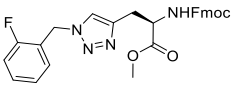
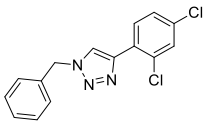
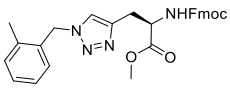
Scheme 2. Synthesis of the small molecule-peptide hybrids via solid phase peptide synthesis, and subsequent small molecule peptide mimic synthesis. DIPEA, diisopropylethylamine; DMF, dimethylformamide; TFA, trifluoroacetic acid; TIPS, triisopropylsilane

Table 1. Binding inhibition IC_{50} of FITC-NoxaB to Mcl-1 of small molecule-peptide hybrid compounds.

	Peptide	Small Molecule	FA IC_{50} (μM)
2	AAQLRRIGD		0.4 ± 0.3
3			0.7 ± 0.8
4			5.8 ± 4.6
5			<100*
6			<100*
7			<100*
8			<100*
9	KVNLRQKLLN		0.1 ± 0.1
10			1.2 ± 9.5
11			3.5 ± 1.5
12			4.3 ± 0.9
13			8.2 ± 1.9
14			8.3 ± 3.6
15			<100*

*Hybrid compound demonstrated binding inhibition less than 100 μM and greater than 10 μM, accurate IC_{50} not determined. Fmoc, 9-fluorenylmethylcarbonyl. IC_{50} determined by non-linear regression of at least three experiments. Errors are the transformed greater extreme of the standard error.

Table 2. Binding inhibition IC_{50} of FITC-NoxaB to Mcl-1 of small molecules and cell growth inhibition of representative compounds towards pancreatic cancer cells lines MiaPaCa-2, BxPC-3, and AsPC-1.

	Structure	FA IC_{50} (nM)	MiaPaCa-2 (μM)	BxPC-3 (μM)	AsPC-1 (μM)
16		33 ± 8	>100	>100	>100
17		102 ± 14	>100	>100	>100
18		186 ± 20	15.19 ± 0.75	29.82 ± 4.15	>100
19		217 ± 58	>100	21.60 ± 8.83	>100
20		249 ± 51	6.57 ± 3.92	5.98 ± 1.14	2.04 ± 0.62
21		1260 ± 142	1.85 ± 3.17	2.81 ± 0.40	>100
22		1680 ± 694	>100	>100	>100
23		1700 ± 230	19.52 ± 1.35	25.21 ± 2.66	10.54 ± 1.53
24		2210 ± 877	2.14 ± 0.56	10.66 ± 2.72	2.28 ± 1.44
25		5500 ± 3923	>100	>100	>100

Fmoc, 9-fluorenylmethylcarbony. IC_{50} determined by non-linear regression of at least three experiments. Errors are the transformed greater extreme of the standard error.

The nature of this approach allows for SAR to be drawn without targeting specific modifications. The most potent compounds, **16** and **17** both possess a heptyl chain which provokes concerns about non-specific hydrophobic events. However, not all compounds synthesised with the heptyl chain demonstrated activity (Table S2). Additionally, the Fmoc-propargylglycine moiety proved effective in several of the identified binders (**18**, **20**, **21**, **23** and **25**), but again was not a feature which was sufficient for binding on its own (Table S2). Concerns over the Fmoc protecting group were considered, but as these compounds are primarily meant as chemical probes or potential leads further development may discover more effective alternatives. Indeed, the Fmoc group is somewhat reminiscent of structural features present in Souers' A-1210477[16d] and Fesik's 2-indole-acysulfonamides,[20] both highly potent and selective Mcl-1 binders. Interestingly, the most potent small molecules were not the combination of the most potent small-molecule peptide hybrids **2** and **9**, as might be expected. The

combination of **2** and **9** demonstrated no appreciable ability to inhibit the interaction of Mcl-1 and FITC-Noxa. Additionally, the small molecule fragment of **9** did not appear in any small molecule which inhibited the Mcl-1/FITC-Noxa interaction. A possible explanation for this phenomenon is that some small molecule fragments, when bound to the peptide fragment, alter the helicity of the peptide, perhaps increasing the binding affinity of the peptide segment, or altering the binding site.[21] Further studies on the power of small molecule peptide hybrids as protein-protein interaction modulators are underway.

It has been shown that NoxaB is a selective Mcl-1 binder.[17] The deployment of the NoxaB peptide as a scaffold for discovering new compounds was envisaged to also impart this selectivity into the new small molecule mimics. To examine this, we employed two *in vitro* fluorescence anisotropy assays with Bcl-2 and Bcl-x_L, using a FITC tagged Bid peptide as our fluorescent marker.[22] Navitoclax (ABT-263) was employed as a positive control, and as an additional indication that the FA assay was performing adequately.[16b] Excitingly, compounds **16-25** and an additional nine compounds (see SI) displayed no appreciable binding to Bcl-x_L or Bcl-2 in our FA assays (Figure 1, see SI), demonstrating a minimum 20 fold selectivity for Mcl-1. To confirm this result was not an artefact, all compounds were re-examined in the Mcl-1, Bcl-2 and Bcl-x_L assays twice more, with the controls of NoxaB peptide and Navitoclax performing as expected. These results also provide some relief about the potential of non-specific hydrophobic events caused by the heptyl chain or Fmoc group.

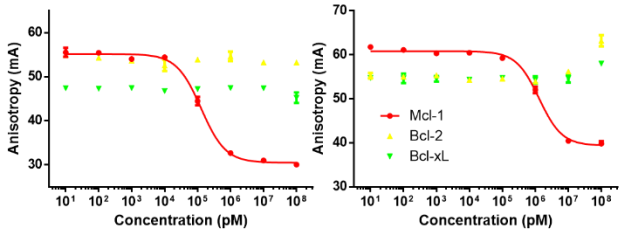


Figure 1. Representative titration of compound **18** (left) and **21** (right) on 5 nM FITC-NoxaB peptide in the presence of 10 nM Mcl-1 protein (red) and 5 nM FITC-Bid peptide in the presence of 30 nM Bcl-2 (yellow) and Bcl-x_L (green), demonstrating no appreciable binding to Bcl-2 and Bcl-x_L.

Compounds **16-25** were examined to determine if they displayed activity towards pancreatic cancer cells which are known to overexpress members of the Bcl-2 family, including Mcl-1. An MTS assay was employed to examine the ability of the compounds to inhibit cell growth and affect metabolism, a potential indicator of cell death. Several compounds, notably **18**, **20**, **21**, **23** and **24**, displayed activity towards the pancreatic cancer cell lines BxPC-3, known to over express Mcl-1, and MiaPaCa-2, which over-expresses both Mcl-1 and Bcl-2. Interestingly, compound **19** only had activity against BxPC-3 cells. The pancreatic cancer cell line AsPC-1, which does not over-express Mcl-1 was also evaluated (Table 2). Two compounds, **18** and **21**, were ineffective against AsPC-1 cells at the concentrations evaluated in our assay, which may suggest they are acting through the inhibition of Mcl-1. The difference in magnitude of activity in cells compared to the *in vitro* FA assay is a commonly observed phenomenon, largely due to cell permeability.[16d, 23] Indeed, some of the more potent compounds in regard to the FA assay showed no activity in the cellular assays, such as compound **17** (IC_{50} = 102 ± 14 nM), suggesting an inability to cross the cell membrane. Additionally, often subnanomolar binding affinities are required for small molecules to compete with high-affinity endogenous ligands.[24] Compounds **18**, **20**, **21** and **23** were highlighted by this assay, and selected for further examination.

To examine if these compounds are impacting on the intrinsic apoptosis pathway, as would be expected if they are binding to Mcl-1 in cells,[25] assays were performed which demonstrate induction of the apoptosis pathway. Compounds **18**, **20**, **21** and **23** demonstrated an increase in caspase-3 activation in BxPC-3 cells 4 hours after treatment, indicated by the cleavage of DEVD-pNa and subsequent increase in optical density at 405 nm.[26] **18** (25 µM, 0.053 ± 0.02), **20** (5 µM, 0.036 ± 0.009), **21** (5 µM, 0.046 ± 0.004) and **23** (25 µM, 0.139 ± 0.06), all displayed an increase in optical density at 405 nm when compared to a vehicle control (DMSO, 0.1%, 0.003 ± 0.005) in this assay (Figure 2, A). Additionally, compounds **18**, **20**, **21** and **23** were shown to induce the externalization of phosphatidylserine on the cell surface,[26] as demonstrated by the binding of annexin-V-FLUOS to BxPC-3 cells resulting in green fluorescence (Figure 2, B).

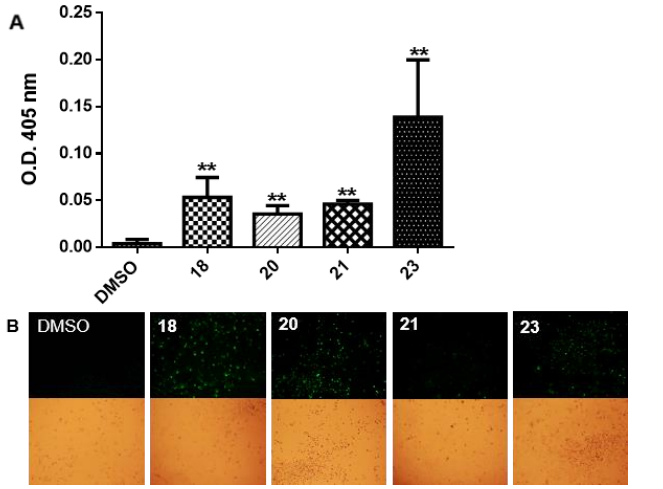


Figure 2. Representative small molecules induce hallmarks of apoptosis. A.Increase of caspase-3 activation in BxPC-3 cells 4 hours after treatment. B.Externalization of phosphatidylserine, a hallmark of apoptosis, was evaluated by exposing BxPC-3 cells to compounds **18** (25 µM), **20** (5 µM), **21** (5µM) and **23** (25 µM) for 4 hours followed by annexin-V-FLUOS. Images were taken at 10x magnification with a GFP filter (top) and white light (bottom).

Utilising the selective NoxaB peptide as a framework, a library of novel Mcl-1 binders which demonstrate selectivity for Mcl-1, has been prepared. Peptide directed binding provided a high percentage of compounds with increased potency when compared to traditional methods of high throughput screening, by the application of a natural peptide framework and simple synthetic manipulations. Recent literature demonstrates that fragment based methods require the screening of approximately 15,000 fragments by NMR and extensive subsequent synthetic manipulations to generate a selective potent Mcl-1 binder.[23] Additionally, high throughput screening has been shown to have a hit rate of 0.2% in recent studies targeting the Bcl-2 family and other prominent cancer PPIs.[27]

The methodology exemplified here represents a significant economic improvement, in both cost and time, when compared to both high throughput screening and fragment based methods and is a powerful new approach to discovering modulators of alpha-helical PPIs. A subset of the identified *in vitro* binders was found to possess activity towards cancer cell lines which overexpress Mcl-1, and induce hallmarks of the apoptosis pathway. It is important to note that these compounds are un-optimised yet still achieved low micromolar cellular activity, exemplifying the power of peptide directed binding to generate potential selective leads swiftly, for challenging targets.

This proof-of-concept study has demonstrated that peptide directed binding is a technique which rapidly identifies new leads for alpha-helical governed protein-protein interactions, and has been effectively exemplified in the Mcl-1/Noxa PPI. Importantly, these compounds demonstrate the ability to mimic the selectivity of the natural scaffold. A recent review on Mcl-1 inhibitors highlights that less than thirty compounds have been reported with comparable selectivity for Mcl-1.[28] Further studies are underway to structurally confirm the binding sites of the hybrid and small molecule compounds generated from peptide directed binding.

It is expected that peptide directed binding is applicable to other alpha-helical PPIs, such as the p53/hDM2 interaction, Bcl-x_L/BIM interaction or HIV gp41 hexameric coiled-coil fusion complex.[29]

Acknowledgements

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