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In silico peptide-directed ligand design complements experimental peptide-directed binding for protein–protein interaction modulator discovery†

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Using the protein–protein interaction of Mcl-1/Noxa, two methods for efficient modulator discovery are directly compared. *In silico* peptide-directed ligand design is evaluated against experimental peptide-directed binding, allowing for the discovery of two new inhibitors of Mcl-1/Noxa with cellular activity. *In silico* peptide-directed ligand design demonstrates an *in vitro* hit rate of 80% (IC₅₀ < 100 μM). The two rapid and efficient methods demonstrate complementary features for protein–protein interaction modulator discovery.

Introduction

Drug discovery for protein–protein interactions (PPIs) has made huge advances in the last decade, making strides to shed the “undruggable” label. The application of fragment based drug discovery^{1–3} and high throughput screening^{4,5} has resulted in many compounds undergoing clinical trials,^{6–9} and the approval of venetoclax in 2016.¹⁰ The methods have proven to be effective, but remain available only to pharmaceutical companies and specially equipped laboratories. In order to address this several innovative techniques have been reported to streamline PPI drug discovery.¹¹ The development of constrained peptides^{12–15} and peptidomimetics,^{16–19} the application of new chemical modalities,^{20–22} and the demonstration of dynamic ligation screening²³ has offered techniques for PPI modulation which utilise the shrewd application of commonly available resources and chemistries.

In an attempt to further improve the economy of drug discovery for PPIs we recently reported peptide-directed binding²⁴ as a method to identify selective small molecule modulators. This technique extends on the REPLACE technique,^{25–27} utilising high affinity peptides as scaffolds for small molecule fragment identification. Using the PPI of apoptosis influencing Mcl-1/Noxa as a paradigm, we took the NoxaB-(75–93)-C75A peptide (AAQLRRIGDKVNLQRKLLN, IC₅₀ 650 nM) and divided it into two half peptides, amino acids 75–84 (AAQLRRIGD) and 85–93

(KVNLRQKLLN, Fig. 1: step 1), each containing two key binding residues (L78, I81, V85, and Q89). Reactive terminals were added to the termini of the half peptides, an alkyne on the C terminus of AAQLRRIGD and an azide on the N terminus of KVNLRQKLLN. These half peptides, with and without reactive termini, possessed no discernible binding affinity for Mcl-1. The reactive termini were used to “click” small molecule fragments to the peptides, with copper catalysed alkyne azide cycloaddition. The binding affinity of the peptide-small molecule hybrids was examined. The fragments which were shown to restore binding affinity in some way emulate the peptide segment they have replaced (Fig. 1: step 2). The small molecule fragments that appeared to emulate amino acids 75–84 were then clicked to the small molecule fragments which emulated amino acids 85–93, providing small molecules with a high probability of emulating the entire NoxaB peptide (Fig. 1: step 3). The choice of small molecule fragments to be clicked to peptides was guided by *in silico* modelling of the covalent reaction, allowing for a high efficiency.

To further improve the efficiency of this method we applied a fully computational peptide-directed ligand design to the PPIs of hDM2/hDMX and p53.²⁸ This process mirrored that of peptide-directed binding, but performed steps 1–3 (Fig. 1) computationally, identifying a number of small molecule triazoles for synthesis. Analogous to our Mcl-1/Noxa example, the crystal structures of a modified p53 peptide bound to both hDM2 and hDMX were modified to generate two half peptides with reactive azide and alkyne termini. *In silico* covalent docking was performed to identify fragments which are likely to restore binding of the semi-peptide. Here the two methods diverge. Instead of preparing the identified peptide-small molecule hybrids, the top 10 azide fragments and top 10 alkyne fragments were combined virtually to identify 100 small molecule triazoles. This library was redocked to the protein target and the top 10 results were chosen for synthesis.

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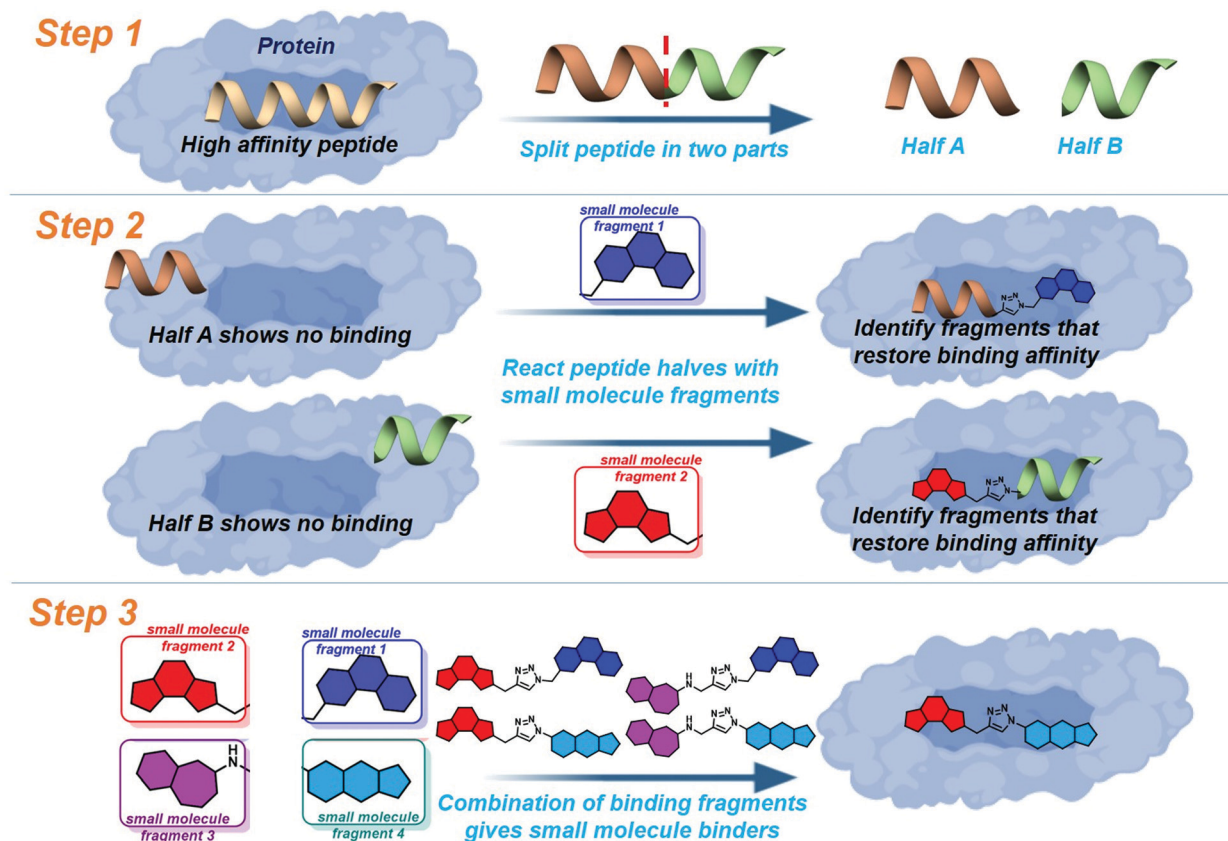


Fig. 1 Schematic of peptide directed binding. The process can be completed experimentally, with the preparation of small molecule–peptide hybrids, identifying fragments which emulate half of the parent peptide. The identified small molecule fragments can be combined to create small molecules which control the target PPI. The process can also be performed virtually, identifying likely small molecule triazole candidates for synthesis.

Our report of *in silico* peptide-directed ligand design²⁸ demonstrated a higher efficiency at discovering small molecule inhibitors of PPIs when compared to the analogous experimental peptide-directed binding.²⁴ The *in silico* method required the preparation of only 20 compounds to obtain a 50% hit rate of compounds which demonstrated an $IC_{50} < 100 \mu M$ in *in vitro* protein fluorescence anisotropy assays. The experimental method revealed 54% of the small molecule compounds prepared demonstrated an $IC_{50} < 100 \mu M$, slightly higher than the *in silico* only route, but also required the preparation of 60 peptide–small molecule hybrids, adding time and cost. The 60 hybrids prepared identified 23 hits (reducing the hit rate to 44% for total compounds synthesised), which suggested 104 possible small molecule triazoles, of which we chose to prepare 35, based on availability and cost of components. It may be by chance that our hit rate was as high as observed, but seems unlikely given the number of compounds observed. The question remained, is one method clearly superior to the other, or are they complementary?

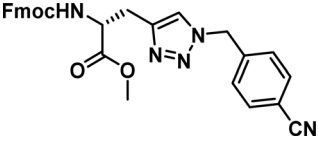
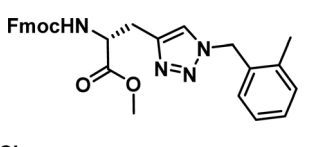
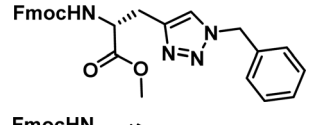
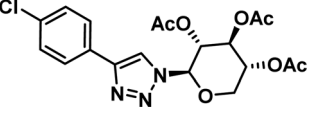
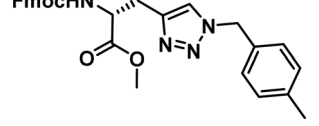
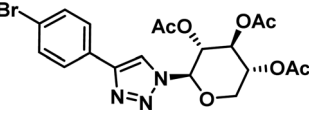
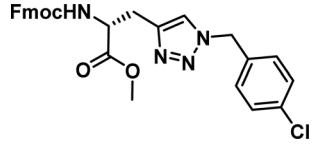
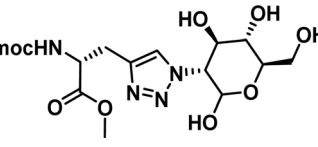
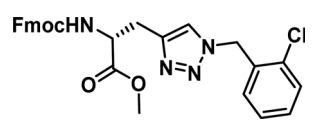
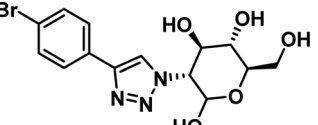
Results and discussion

To examine this, we performed *in silico* peptide-directed ligand design on the Mcl-1/Noxa interaction, allowing for a direct comparison of the experimental *versus* computational approach.

The crystal structure of Mcl-1/NoxaB (PDB ID 2NLA²⁹) was modified to generate the virtual reactive semi peptide AAQLRRIGD-propargylglycine and covalent docking^{30,31} was used to model copper catalysed azide alkyne cycloaddition *in silico* allowing us to identify the top ten³⁰ synthetically viable small molecule azides fragments, from a commercially available library of 214 azides, which fit into the site vacated by the other section of peptide. Similarly, the crystal structure was modified to generate α -azidoamide-KVNLRQKLLN, and covalent docking was performed to identify small molecule alkyne fragments from a commercially available library of 869 alkynes. To avoid bias the library was taken directly from the Merck catalogue of available reagents. The top 10 azide and top 10 alkyne fragments were used to create a virtual library of 100 triazoles which was docked to Mcl-1 in an induced fit manner to score and rank the library. The library was also evaluated to predict ADME properties (SwissADME evaluation predicting lipophilicity, water solubility, pharmacokinetics, drug-likeness (Lipinski, Ghose, Veber) and PAINS and lead-likeness screen),³² to help ensure synthetic effort was most likely to generate results. The top 10 results were chosen for synthesis following published procedures with $CuSO_4$ and sodium ascorbate in a *t*BuOH/ H_2O mix,²⁴ followed by purification with reverse phase HPLC. Synthesised compounds were evaluated in the FITC-NoxaB Mcl-1 competitive binding fluorescence anisotropy (FA) assay (Table 1 and Fig. 2A).²⁴



Table 1 IC₅₀ values for the inhibition of the binding of FITC-NoxaB to Mcl-1 of small molecules^a

Structure	FA IC ₅₀ (μM)	Structure	FA IC ₅₀ (μM)
	0.17 [0.15–0.19]		3.38 [2.56–4.47]
	0.36 [0.29–0.45]		4.39 [3.95–4.87]
	0.62 [0.47–0.80]		7.03 [6.20–7.97]
	1.01 [0.89–1.15]		> 100
	1.14 [0.84–1.52]		> 100

^a IC₅₀ values determined by non-linear regression of at least three independent experiments (see ESI). Errors are 95% confidence intervals (CI). Fmoc, 9-fluorenylmethylcarbonyl.

The NoxaB peptide (AAQLRRIGDKVNLRLQKLLN) was employed as a positive control and to ensure the FA assay was performing adequately. Surprisingly, of the ten compounds prepared eight (80%) demonstrated binding in our assay, with an IC₅₀ < 100 μM. Additionally, three of these compounds (1–3) demonstrated IC₅₀ values less than 1 μM. Selectivity for the Mcl-1/Noxa was evaluated by also examining the compounds in assays for Bcl-2/Bim, Bcl-xL/Bim, and Nrf2/Keap1. The ten compounds demonstrated excellent selectivity, with no appreciable inhibitory effect in these assays.

This very high hit rate demonstrates the power of peptide-directed ligand design for PPI modulators. Analysis of the compounds shows that four compounds were identified by experimental peptide-directed binding (1, 2, 4 & 6),²⁴ with four new Mcl-1 binders identified with the *in silico* approach (3, 5, 7 & 8). Three of the top four binders (1, 2 & 4) were identified by the experimental peptide-directed binding, suggesting both experimental and computational methods are likely to find the most efficacious compounds. However, 1–6 have very similar structures, with the Fmoc-D-propargylglycine unit cyclised with a benzylazide, all of which demonstrated IC₅₀ values < 10 μM. The *in silico* method highlighted four new small molecule fragments which had not been highlighted in the experimental peptide-directed binding methodology²⁴ (azide section of 3, 7, 8, and the alkyne section of 7 and 8). The binding of compounds to Mcl-1 was validated with a thermal shift assay³³ using the hydrophobic dye Sypro orange.³⁴ Compound 1–8 demonstrated the ability to increase the melting temperature of Mcl-1 compared to the DMSO vehicle control (Fig. 2B).

The compounds which demonstrated activity in the *in vitro* protein assays were evaluated in the MTS anti-proliferation assay,³⁵ against pancreatic cancer cells lines. BxPC3 cells, which are dependent on Mcl-1, MiaPaCa2 cells, which are dependent on Mcl-1 and Bcl-2, and AsPC-1 cells, which do not overexpress Mcl-1,^{36–39} were treated with compound 1–8 (Table 2). Compound 5–8 showed no antiproliferative effect in all evaluated cell lines. Compound 1 and 2 demonstrated antiproliferative effects on all evaluated cell lines. Interestingly, compound 3 and 4 demonstrated efficacies in BxPC3 and MiaPaCa-2 cells, but no effect on Mcl-1 independent AsPC-1 cells, perhaps suggesting an Mcl-1 selective mode of action. 3 and 4 also suggest a *para* substituent of a certain size on the phenyl portion may offer selectivity. Compound 9 and 10 were highlighted by the ADME evaluation as unlikely to demonstrate cellular activity (*c log P* of 0.55 and –0.03 respectively), but were prepared nonetheless due to their high docking ranking. These results suggest that the subsequent ADME evaluation will offer increased efficiency if heeded.

A tetramethylrhodamine ethyl ester (TMRE) assay was employed to evaluate if compounds generated mitochondrial membrane depolarization, one of the first signs of Mcl-1 inhibition (Fig. 2C).^{40,41} BxPC-3 cells were incubated with compound 1–4 or positive control, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), followed by TMRE. TMRE readily accumulates in the mitochondria resulting in a fluorescent signal. Depolarized mitochondria result in reduced fluorescence. Compound 1–4 showed significantly reduced fluorescence after 2 h of incubation, indicating depolarized mitochondria membrane and apoptosis.



synthesis required to generate selective modulators. Both of these techniques offer PPI researchers a rapid and efficient method to generate selective modulators of α -helical PPIs.

Conflicts of interest

There are no conflicts to declare.

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