Video Article

Development of Inhibitors of Protein-protein Interactions through REPLACE: Application to the Design and Development Non-ATP Competitive CDK Inhibitors

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Abstract

REPLACE is a unique strategy developed to more effectively target protein-protein interactions (PPIs). It aims to expand available drug target space by providing improved methodology for the identification of inhibitors for such binding sites and which represent the majority of potential drug targets. The main goal of this paper is to provide a methodological overview of the use and application of the REPLACE strategy which involves computational and synthetic chemistry approaches. REPLACE is exemplified through its application to the development of non-ATP competitive cyclin dependent kinases (CDK) inhibitors as anti-tumor therapeutics. CDKs are frequently deregulated in cancer and hence are considered as important targets for drug development. Inhibition of CDK2/cyclin A in S phase has been reported to promote selective apoptosis of cancer cells in a p53 independent manner through the E2F1 pathway. Targeting the protein-protein interaction at the cyclin binding groove (CBG) is an approach which will allow the specific inhibition of cell cycle over transcriptional CDKs. The CBG is recognized by a consensus sequence derived from CDK substrates and tumor suppressor proteins termed the cyclin binding motif (CBM). The CBM has previously been optimized to an octapeptide from p21Waf (HAKRRIF) and then further truncated to a pentapeptide retaining sufficient activity (RRLIF). Peptides in general are not cell permeable, are metabolically unstable and therefore the REPLACE (REplacement with Partial Ligand Alternatives through Computational Enrichment) strategy has been applied in order to generate more drug-like inhibitors. The strategy begins with the design of Fragment ligated inhibitory peptides (FLIPs) that selectively inhibit cell cycle CDK/cyclin complexes. FLIPs were generated by iteratively replacing residues of HAKRRLIF/RRLIF with fragment like small molecules (capping groups), starting from the N-terminus (Ncaps), followed by replacement on the C-terminus. These compounds are starting points for the generation of non-ATP competitive CDK inhibitors as anti-tumor therapeutics.

Video Link

The video component of this article can be found at https://www.jove.com/video/52441/

Introduction

In this article, a case study of applying the REPLACE (Replacement with partial ligand alternatives using computational enrichment) strategy to convert peptidic inhibitors of protein-protein interactions into more pharmaceutically relevant molecules is described[1-3]. While PPIs represent a rich but underexploited source of potential drug targets, existing methodologies are largely insufficient to make these widely accessible. Current strategies including fragment based design[4], high-throughput screening[5] and stapled peptides[6] have provided advances, however these are in many cases ineffective. As a result, more progress and more efficient approaches are required. REPLACE has been fully validated in the development of kinase inhibitors that have improved drug-like properties and have potential for further development as anti-tumor therapeutics. This strategy is exemplified in the development of non-ATP inhibitors of cell cycle CDKs and involves as follows: 1) obtaining 3D structural information on the interactions of HAKRRLIF/RRLIF with the cyclin binding groove; 2) determining the important binding determinants for peptide interaction; 3) truncation of the peptide N-terminus containing one or more binding determinants; 4) computational identification of potential small molecule alternatives (partial ligand alternatives, PLAs) for the truncated portion of the peptide and which retain key interactions of the parent peptide; 5) synthesis or commercial sourcing of PLAs predicted to bind avidly with the sub site previously occupied by the deleted peptide residue(s); 6) synthesis of FLIPs through ligation of the best PLAs to the truncated peptide using solid phase synthesis; 7) testing of FLIPs in an *in vitro* binding or functional assay (fluorescence polarization in the CDK/cyclin context) followed by further characterization in a cell viability assay. A schematic representation of REPLACE strategy is shown in **Figure 1**. In this article, iterations of the REPLACE strategy are discussed and the application to CDK2/cyclin A described in detail. CDKs are believed to be directly or indirectly deregulated in the majority of tumors and are therefore considered appropriate cancer drug targets[7]. CDKs require association with cyclins for full activation and subsequently phosphorylate key proteins involved in cell cycle regulation[8]. The two major groups of CDKs are the isotypes that control cell cycle checkpoints [G1/S (CDK4/Cyclin D, CDK6/cyclin D and CDK4/cyclin E), S phase (CDK2/cyclin A) and G2/M (CDK1/cyclin B)] and the regulators of RNA polymerase through phosphorylation (CDK7/cyclin H, CDK8/cyclin C, CDK9/cyclin T). A key step in S phase progression occurs when the E2F1 transcription factor forms a complex with the DP protein which then binds to DNA and initiates gene transcription. CDK2/cyclin A is required

to neutralize E2F1 transcriptional activity through phosphorylation thereby leading to release of the E2F1-DP complex and its subsequent degradation. Inhibition of CDK2/cyclin A is believed to maintain E2F1 in its DNA bound state leading to persistent activation. The resultant level of E2F-1 activity will surpass the threshold required to induce p53 independent apoptosis therefore suggesting a therapeutic strategy. Due to deregulated p53 and pRb pathways, high levels of E2F-1 frequently occur in cancer cells and inhibition of CDK2/cyclin A should lead to selective apoptosis in tumors and can be considered as a validated cancer target[7].

Clinically investigated CDK inhibitors target the highly conserved ATP binding site leading to cross reactivity among the greater than 500 protein kinases in the human kinome and potentially giving rise to side effects and toxicity[9]. An alternate approach is non-ATP competitive inhibition by targeting substrate recruitment through the CBG present on cyclin positive regulatory subunit and which is therefore distinct and distant from ATP binding site[10,11]. The CBG is primarily a hydrophobic groove present in cyclin A, cyclin D and cyclin E and has been shown to recognize a consensus sequence found in substrates and tumor suppressors. As an isolated peptide, the cyclin binding motif (CBM) binds to the CBG and has been shown to inhibit kinase activity of the cell cycle CDKs. The CBM has been optimized to an octapeptide (HAKRRLIF, CDK2/cyclin A IC_{50} 0.07±0.02 μM , CDK4/cyclin D, IC_{50} 0.88±0.34 μM) and furthermore truncated to a pentapeptide representing a good compromise between molecular weight for drug-likeness and potency (RRLIF, CDK2/cyclin A IC_{50} 1.01±0.17 μM, CDK4/cyclin D, IC_{50} 25.12±2.97 μM)[12,13]. The CBGs consist of a large primary and smaller secondary hydrophobic pocket which are bridged by an acidic region (includes Glu220, Glu224 and Asp283). The key binding determinants of HAKRRLIF include the interaction of Ala2 with the secondary hydrophobic pocket, ion pairing and hydrogen bonds of Lys3, Arg 4 and Arg5 with the acidic region and a high degree of complementarity of Leu6 and Phe8 with the primary lipophilic site. In addition, numerous hydrogen bonds are contributed from the peptide backbone while Ile7 acts as a spacer residue allowing optimal contact with the primary pocket. The binding mode and interactions of HAKRRLIF with CBG is shown in **Figure 2**.

Targeting the CBM/CBG protein-protein interaction will inhibit kinase activity of CDK2/cyclin A, CDK2/cyclin E & CDK4/cyclin D and this should trigger E2F1 mediated apoptosis of cancer cells while not affecting normal cells[7]. Although CBM derived peptides are effective inhibitors of cell cycle CDKs, it is unlikely that they will be useful as drugs due to their metabolic instability and general lack of cell permeability. To this end, we have applied the REPLACE strategy in order to convert these potent peptidic inhibitors into more drug-like compounds for further development of anti-tumor therapeutics exploiting deregulated E2F1 through CDK2/cyclin A inhibition. The following protocol summarizes work that has been completed in the application of REPLACE to the cyclin groove. In the first instance, drug-like capping group replacements for the N-terminal tetrapeptide of HAKRRLIF were identified. Furthermore improvements in these groups were investigated in an additional validation study for REPLACE. Representative results from these studies are also presented.

Protocol

1. Computational Identification of Potential Small Molecule Capping Groups

Note: In principle, a variety of docking or pharmacophore search methods can be used to predict potential capping groups. The main purpose of computational studies in REPLACE is to identify small molecules that retain the features and interactions of the amino acids that are substituted.

1. Validation of the LigandFit docking protocol[14]

Note: In previous studies, the docking method (LigandFit[15], a module in the molecular modeling program suite, Discovery Studio 3.0) was validated to ensure that this algorithm is sufficient to reproduce binding modes of known Ncaps and to show that the results obtained for unknown compounds are predictive[14].

1. Using the following steps (1.2 – 1.5), identify optimal parameters for LigandFit as follows: PLP1 energy grid for pose generation, minimization of generated poses and the PLP1 scoring function for the analysis of poses.
2. In order to limit the conformations obtained and to position ligands appropriately for covalent bond formation in the capped peptide, set the indole nitrogen and amide nitrogen atoms of Trp217 and Gln254 respectively as interaction filters for hydrogen bonding.
3. Design and dock a library of fragment alternatives into the CDK2/cyclin A receptor (2UUE). Prioritize potential capping groups for synthesis and commercial sourcing based on PLP1 scoring, interactions with interaction filters and complementarity with the CBG. The various steps required for LigandFit docking are as follows.

2. Preparation of receptor binding site[14]
    1. Import the CDK2/cyclin A crystal structure (pdb id: 2UUE) into a visualization window in the software. This structure contains the previously identified FLIP 5-methyl-1-phenyl-1H-1,2,4-triazole-3-carboxamido(3,5-DCPT)RLIF bound to the cyclin groove (**Figure 3**).
    2. Delete or keep in place the residues RLIF (C-terminus). When the peptide sequence is retained, use the N-terminal amino group of the peptide as an interaction filter for the ligand. From the “Receptor-ligand interactions” tools, create a sphere around the N-cap 3,5-DCPT from Show/Hide site sphere. The sphere is created to define the active region in the binding site where the ligand-receptor interactions are allowed to occur during binding.
    3. Define the binding site further from the “find site as volume of selected ligand” and delete the ligand 3,5-DCPT from the protein. Carry out this step to define the volume of binding within the sphere created.
    4. Set the indole nitrogen and amide nitrogen atoms of the residues Trp217 and Gln254 as interaction filters for hydrogen bonding. Set the interaction filters so that only those poses that interact with the atoms of set residues will be filtered during the docking process therefore preserving important interactions in the docked poses.

3. Preparation of ligands[14]
    1. Design a library of 100 potential capping groups based on criteria including molecular weight less than 500, presence of a carboxylate group for ligation with the truncated peptide and inclusion of appropriate hydrophobic groups (substituted phenyl group) for van der Waals interaction in the secondary pocket as shown in **Table 1**.

2. Select heterocyclic rings for mimicking peptide hydrogen bonding interactions with Trp217 and substituents included for replacing ion-pairing interactions of basic side chains of the HAKRRLIF. Dock the ligands as respective aldehyde molecules so that the carbonyl oxygen can interact with the amide nitrogen atom of Gln254 similar to peptide backbone in the parent peptide (**Table 1**).
3. Prior to docking, minimize designed ligands to a low energy conformation and prepare in an appropriate ionization state using the "Prepare ligands" protocol. Draw and export these potential N caps in the .sdf file format in ChemDraw for a spreadsheet prior to being imported into the software.
   Note: Prepare ligands protocol is used to minimize all the ligands to be docked to their lowest energy states and the ionization charges were applied to the applicable atoms. Default parameters are used for this step.

4. Docking of the ligands into the Cyclin A Groove[14]
   1. Select the LigandFit routine from receptor-ligand interactions protocol set of the software. Use the receptor and the prepared ligands as input for this protocol.
   2. For these runs select PLP1 as energy grid, specify that poses be energy minimized and that the number of generated poses as 10. Leave all the other parameters at the default values.
      Note: LigandFit is a docking method which can identify and generate poses of high complementarity and potential affinity with the active site of a protein using a shape based comparison filter which is combined with a Monte Carlo conformational search algorithm. The energy grid used by the docking program is the force field for generating ligand-receptor interactions and potential poses. PLP1 is piecewise linear potential which specifically prioritizes hydrogen bonding interactions and where the PLP atom type is assigned for each non-hydrogen ligand atom or non-hydrogen receptor atom.

5. Analysis of results
   1. In the first instance, display poses in the visualizer program and then sort by descending values of the PLP1 score. Use scoring functions such as PLP1 to estimate the binding affinity of a docked ligand based on a candidate ligand pose geometry and non-covalent interaction with the target receptor structure.
      Note: Here, the PLP1 scoring function was found to give the best results as it includes an estimation of hydrogen bonding.
   2. Analyze visually the top 25% of the scored poses for 1) superimposability with the known capping group (having a relative mean square deviation (RMSD) value less than 2 Å), 2) fulfilled interaction filters and 3) visual complementarity with the cyclin groove. It is accepted in that a correctly docked pose (compared to an experimental structure) has an RMSD value of < 2 Å.
   3. Examine poses for visual complementarity, which is defined as having efficient filling of the binding pocket in a manner that is consistent with known structure-activity relationships. Interaction filters are set to include atom restraints that require intermolecular contacts known to be critical for binding and/or are required to position the potential capping group in the correct geometry for amide bond formation. Three examples of docked poses of potential N-capping groups making hydrogen bonds with interaction filters are shown in **Figure 4**.

2. Synthesis and Characterization of Potential *N*-capping Groups

1. Synthesis of N-capping groups.
2. For synthesis, use all commercial starting materials, solvents and reagents as obtained. Synthesize potential N-capping groups by conventional synthetic organic chemistry (**Figure 5**[12,13] for an example).
3. Perform thin layer chromatography (TLC) on silica gel for monitoring reactions.
   1. Dissolve the starting material and reaction mixture (1 mg) in the mobile phase and spot them on the TLC plate using capillary tubes.
   2. Place the TLC plate in to the chamber containing mobile phase (ethyl acetate and hexane at a 35:65 ratio). Once the mobile phase reaches 90% of the TLC plate, remove and air dry the plate.
   3. Use UV light to detect the starting material and reaction mixtures as visible spots. Calculate the R_f of all the spots (ratio of distance travelled by the spot and the mobile phase).
      Note: The reaction is complete when there is no spot seen at the R_f of the starting material.

4. After the completion of reaction work up the reaction mixture by placing in a separatory funnel and washing with aqueous acid or base solution as appropriate. Collect the organic solvent, evaporate it in rotary evaporator and dry the crude product obtained under vacuum.
5. Dissolve 500 mg of crude product in 3-5 ml of suitable solvent and add to a 1 g of silica or RP18 samplet and dry under air. Place the samplet containing the crude product in the silica/ RP SNAP flash cartridges and purify the crude material using automated high performance flash chromatography (according to manufacturer's protocol) employing a SNAP 100 g column with a gradient run starting from 6% ethyl acetate: 94% hexanes to 50% ethyl acetate and 50% hexanes over 15 column volumes.
6. Dry the purified product collected in solvent from flash chromatography using a rotary evaporator by evaporating all the solvent to dryness and further dry the product under vacuum to remove all the residual solvents. Perform characterization of the purified product by NMR, MS and analytical HPLC.
   1. For ^{1}H NMR and ^{13}C NMR, weigh approximately 10 mg of the purified sample, dissolve it in an appropriate deuterated solvent, and transfer the contents to a dry NMR tube for recording the NMR spectra[2,14].
   2. Record the ^{1}H NMR and ^{13}C NMR spectra with a high field NMR Spectrometer (minimum 300 MHz). Acquire mass spectra using a QTOF (Tandem quadruple-1 time of flight mass spectrometer), electrospray ionization (ESI) or a VG 70S (Double-focusing magnetic sector mass spectrometer, EI)[2,14].
   3. Analyze the purity of products by HPLC with a diode-array detector and equipped with a C18 (2) 100 A, 250 x 4.6 mm, 5 µm column for analysis. Use a gradient run starting from 100% water (0.1% trifluoroacetic acid) to 60% acetonitrile (0.1% trifluoroacetic acid) over 30 min and hold the gradient for 4 min. Extract the chromatograms at 226 and 254 nm[2,14].
      Note: Analytical purities of evaluated compounds were > 95%.

3. Solid Phase Synthesis for the Generation of FLIPs [2]

1. Synthesis of N-capped FLIPs
    1. Assemble N-capped peptidic compounds through standard solid-phase synthesis methods using the following steps.
        1. Activate 5 equivalents of the C-terminal amino acid (Fmoc-Phe) in 4.4 equivalents of *O*-Benzotriazole-*N,N,N',N'*-tetramethyl-uronium-hexafluoro-phosphate (HBTU, 221.93 mg) in 2 ml of DMF for 5 min. Load the Fmoc-Phe HBTU mixture onto the Rink resin using 6 equivalents of diisopropylethyl amine (DIPEA, 103.13 mg) for 1 hr at RT.
        2. Remove the Fmoc protecting group from the C-terminal residue using 20% piperidine in 3 ml of DMF for 10 min. Couple subsequent amino acids (*e.g.*, Fmoc-Ile, Fmoc-Leu, Fmoc-Arg-pmc) step by step. At each step, couple 5 equiv of the next amino acid using 6 equivalents of DIPEA (103.13 mg) and 4.4 equivalents of HBTU (221.93 mg) in 2 ml of DMF for 1 hr at RT.
        3. Apply wash cycles (5 x 10 ml of DMF + 5 x 10 ml of DCM) after amino acid coupling and Fmoc deprotection steps described above. After peptide assembly, couple N-capping groups using 6 equivalents of DIPEA (103.13 mg) and 4.4 equivalents of HBTU (221.93 mg) in 2 ml of DMF for 1 hr at RT.
        4. Upon completion of peptide assembly, treat the reaction mixture with 2 ml of the cleavage mixture (90:5:5 TFA/H_2O/TIPS) O/N to remove side chain protecting groups, and cleave FLIPs from the resin. Triturate the resulting product with cold diethyl ether to precipitate and if necessary concentrate in a rotary evaporator.

2. Synthesis of N-capped-C-capped FLIPs
    1. Weigh and dissolve the N-terminally capped and protected Arg-Leu peptide (16.1 mg, 0.02 mmol) in dichloromethane, and add [4-(3-chlorophenoxy)pyridin-2-yl]methanamine (C-cap) along with *O*-benzotriazole-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU; 16.7 mg, 0.02 mmol) and *N,N*-diisopropylethylamine (DIPEA; 6.5 mg, 0.02 mmol).
    2. Stir and monitor the reaction at RT until TLC/HPLC indicates the complete consumption of starting material. After the completion of the reaction, concentrate the crude reaction mixture using a rotary evaporator and then partition between ethyl acetate and water.
    3. Separate the aqueous and organic layer; wash the organic layer with 1 N NaOH, 1 N HCl, and brine. Dry the organic layer with about 1 g of sodium sulfate and concentrate the product in rotary evaporator. Finally treat the product O/N with the cleavage mixture (95:2.5:2.5 trifluoroacetic acid/H_2O/TIPS) for deprotection.
    4. Triturate the resulting product with cold diethyl ether and if necessary concentrate in rotary evaporator to complete dryness in order to remove all the solvent. Further dry the product under vacuum to remove all the residual solvents.

3. Purification and characterization of FLIPs
    1. Purify crude FLIPs using semi preparative reverse-phase HPLC methods. Lyophilize the purified FLIPs and characterize their molecular mass using mass spectrometry[2,14].
    2. Determine the analytical purity of the FLIPs by HPLC with a diode-array detector and equipped with a C18 (2) 100 A, 250 x 4.6 mm, 5 µm column. Use a gradient method starting from 95% H_2O (0.1% trifluoroacetic acid)/5% acetonitrile (0.1% trifluoroacetic acid) to 35% H_2O (0.1% trifluoroacetic acid)/ 65% acetonitrile (0.1% trifluoroacetic acid) over 30 min and hold the gradient for 4 min. Extract chromatograms at 226 and 254 nm.

4. Fluorescence Polarization Binding Assay for the Determination of Competitive Binding [2,14]

1. Perform the assay in black 384-well (138 µl) plates using the following procedure.
2. Dilute the samples, tracer peptides (4 nM), and kinase complexes (CDK2/cyclin A — 18 µg/ml, CDK4/cyclin D — 37 µg/ml) to required concentrations in assay buffer (25 mM HEPES pH 7, 10 mM NaCl, 0.01% Nonidet P-40, 1 mM dithiothreitol (DTT).
3. To each well of the 384-well plate, add: 5 µl of CDK4D1 or CDK2CA (0.3 µg/well purified recombinant human kinase complex), 5 µl compound solution, 5 µl of 30 nM tracer peptide (fluoresceinyl-Ahx-Pro-Val-Lys-Arg-Arg-Leu-(3ClPhe)-Gly or fluoresceinyl-Ahx-Pro-Val-Lys-Arg-Arg-Leu-Phe-Gly tracer peptide). Use 5 µl of each DMSO (6%), buffer, tracer and 5 µl DMSO (6%), kinase complex, tracer as control wells
4. After the addition of the all the components, centrifuge the plate at 500 rpm for 1 min (41.16 x g) at RT and then incubate the plate with agitation for 45 min at RT. Using a multimode plate reader and detector fitted with 485 nm/535 nm excitation/emission filters and a dichroic mirror read the fluorescein intensity of each well in the plate.
5. Calculate the relative mean polarization (mp) for all the test samples using the equation showing below. Determine IC_{50} values by logarithmic regression by correlating relative mps and testing concentrations.

$$\text{Relative mp} = \frac{\text{mP(compound)-mP(DMSO, protein, tracer)}}{\text{mP(DMSO, protein)-mP(DMSO, protein, tracer)}}$$

Representative Results

The interactions of HAKRRLIF with the cyclin groove are shown in **Figure 2**. The peptide residues that represent the key binding determinants include Ala2, Arg4, Leu6 and Phe8 with other residues providing smaller contributions[12,13,18]. In this case study the REPLACE strategy has been utilized in order to find fragment alternatives for residues in the N-terminal tetrapeptide of HAKRRLIF, primarily mimicking the interactions of Ala2 and Arg4. A library of potential Ncap fragments (**Table 1**) was constructed based on the criteria. Each molecule was docked into the vacated binding site created by truncation of the N-capping group from its crystal structure with cyclin A (PDB ID: 2UUE). Potential ligand alternatives were selected based on pose analysis and PLP1 scoring as a prediction of affinity (examples of docked poses are shown in

Figure 4). The synthesis of a confirmed Ncap (1-(3-chlorophenyl)-5-methyl-1H-1,2,4-triazole-3-carboxylic acid) is shown in **Figure 5** where this molecule was generated in three steps and purified by automated flash chromatography (**Supplementary Figure 1**). The ^{1}HNMR, ^{13}CNMR, MS and HPLC characterization data of a representative compound 1-(3-chlorophenyl)-5-methyl-1H-1,2,4-triazole-3-carboxylic acid are shown in **Supplementary Figures 1-5** respectively. The synthesis of N-capped FLIPs and N-capped-C-capped FLIPs are represented in **Figures 7** and **8** and the characterization data are shown in **Table 2**. The binding affinity for both CDK2/cyclin A and CDK4/cyclin D was determined using the described FP assay and the results are shown in **Table 3**. Of the compounds tested, FLIPs containing triazole based N-caps were determined to have the greatest affinity for CDK2/cyclin A and CDK4/cyclin D and representative compounds were tested in cell based assays. As a whole, FLIP molecules identified were found to be more drug like, and to retain most of the interactions of the HAKRRLIF octapeptide. Capped tetrapeptides possessed lower potency compared to HAKRRLIF however were of comparable activity to the RRLIF pentapeptide (**Tables 3** and **4**). These results show that the compounds obtained had improved drug likeness but this was obtained at the cost of somewhat compromised binding affinity.

The anti-proliferative activity these CGI ligands was evaluated and cellular IC_{50}'s below 30 μM were observed in U2OS and DU145 cancer cell lines.

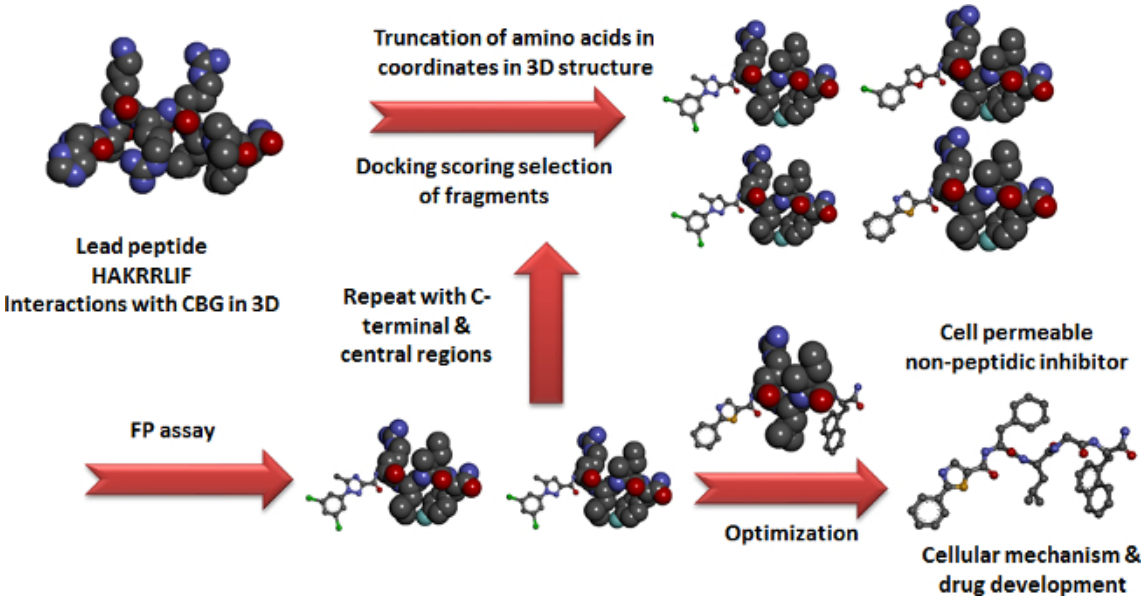


Figure 1. Overview of the REPLACE strategy. Please click here to view a larger version of this figure.

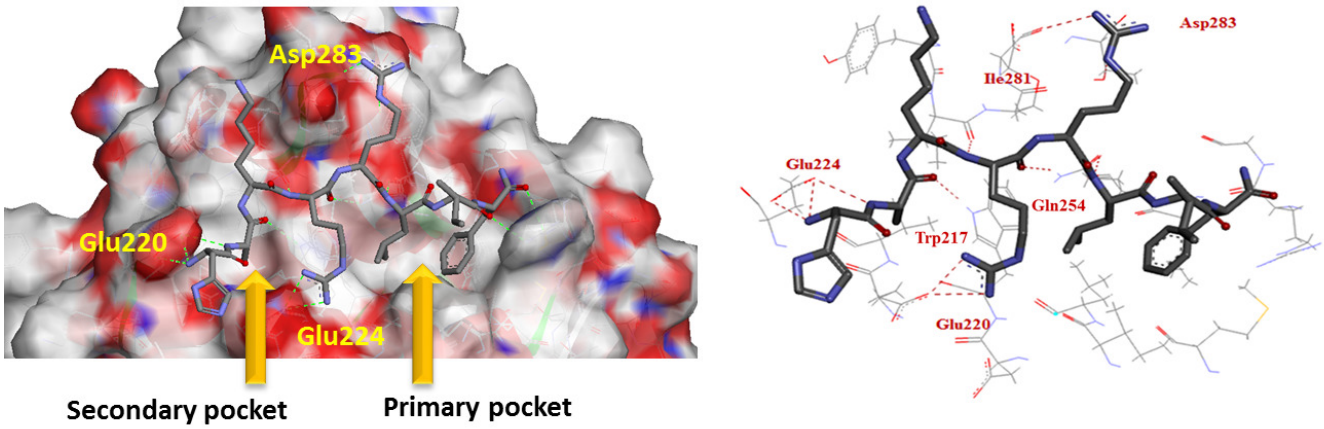


Figure 2. Binding and interactions of HAKRRLIF in CBG. Left panel: Modeled structure of HAKRRLIF bound to the CBG. The cyclin groove consists of two hydrophobic pockets — a larger primary pocket (right, occupied by Leu6 and Phe8) and a smaller secondary pocket (left, occupied by Ala2) and which are bridged by acidic residues depicted in red. Right panel: Other important interactions include hydrogen bonding contacts of the peptide backbone (with Trp217, Gln254, Ile281), and ion-pairing interactions of the three basic residues of the peptide (with Glu220, Glu224, Asp283). Please click here to view a larger version of this figure.

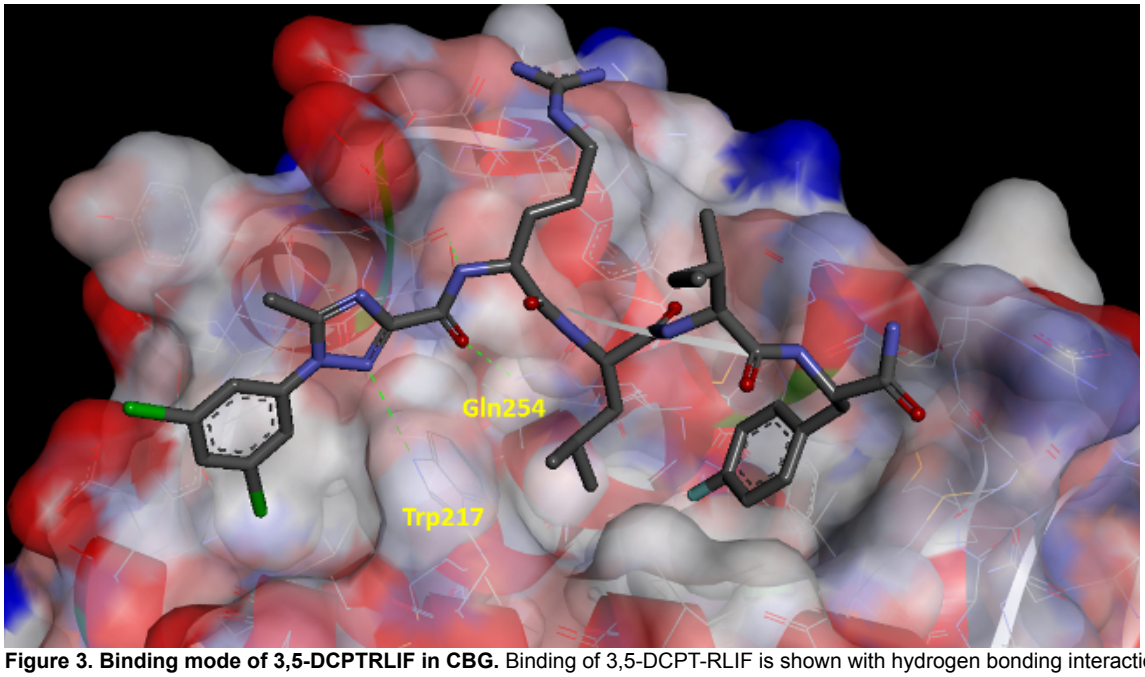


Figure 3. Binding mode of 3,5-DCPTRLIF in CBG. Binding of 3,5-DCPT-RLIF is shown with hydrogen bonding interactions (depicted by green dotted lines) with sidechain atoms of Trp217 and Gln254**.** Please click here to view a larger version of this figure.

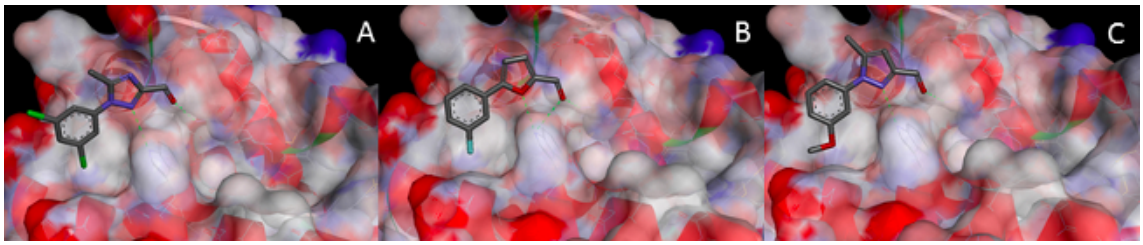


Figure 4. Selected docking results. Docked poses of 3 representative N-capping groups are shown. Each of these compounds make H-bonds with the interaction filter atoms of Trp217 and Gln254 and as depicted by green dotted lines. The phenyl substituent makes van der Waals interactions with the secondary hydrophobic pocket. Please click here to view a larger version of this figure.

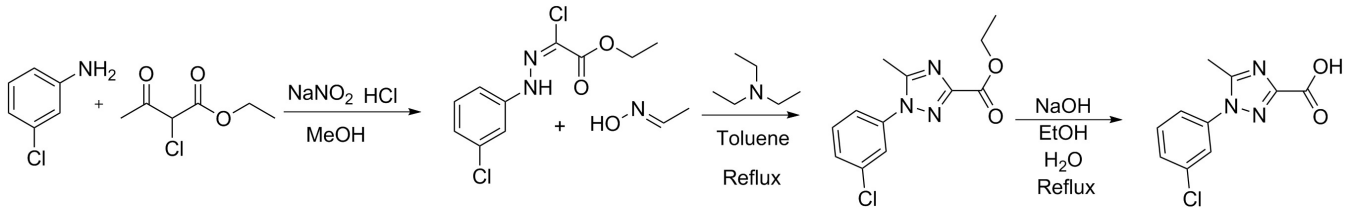


Figure 5. Synthesis of 1-(3-chlorophenyl)-5-methyl-1H-1,2,4-triazole-3-carboxylic acid. Please click here to view a larger version of this figure.

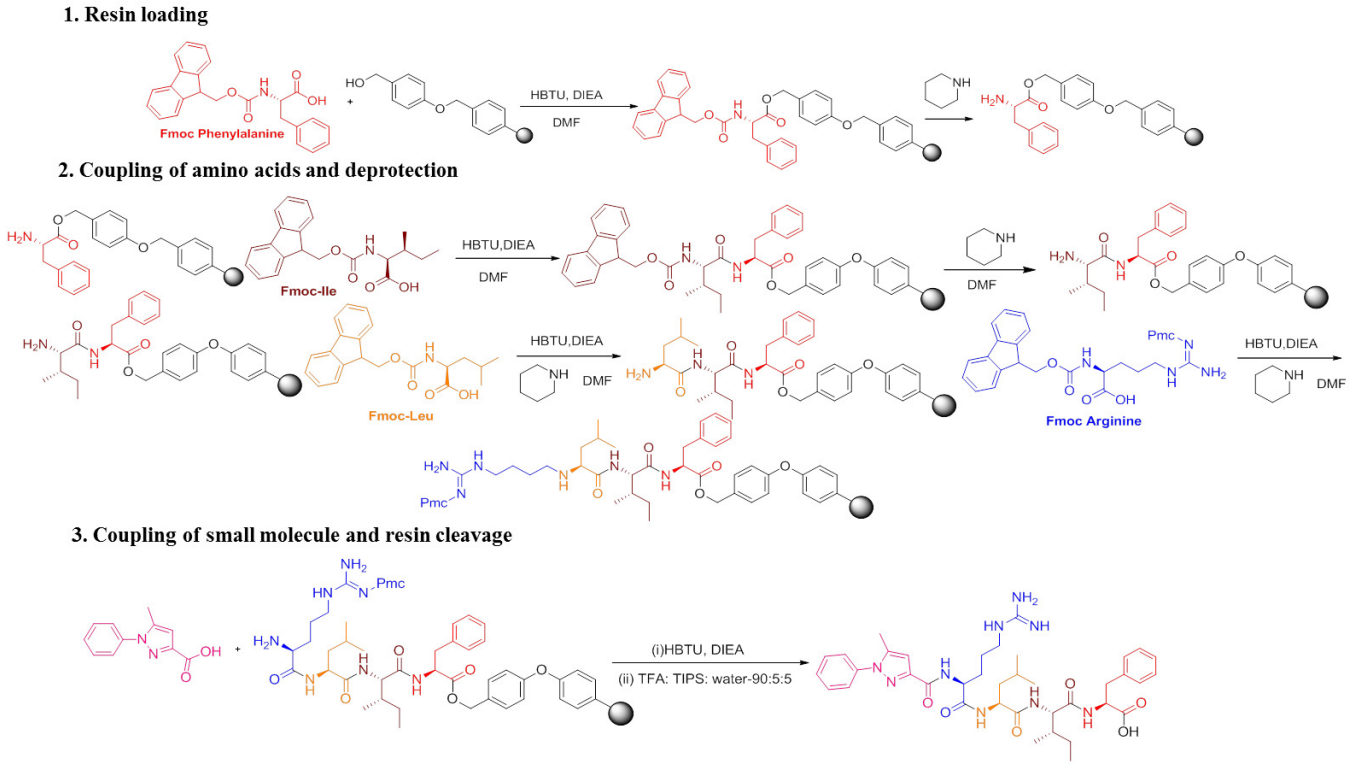


Figure 6. Synthesis of N-capped FLIPs by solid phase synthesis. Please click here to view a larger version of this figure.

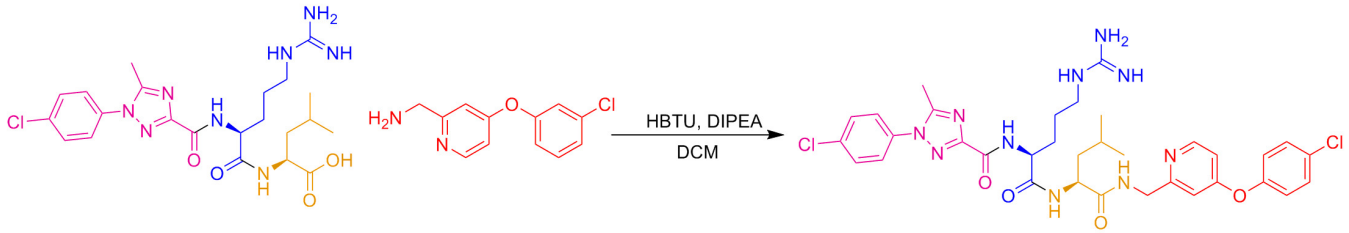
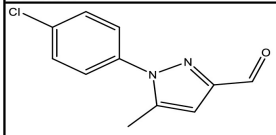
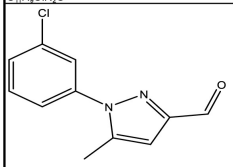
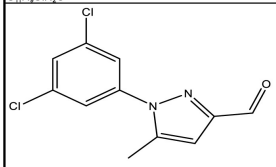
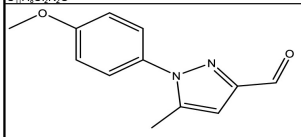
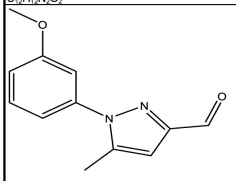
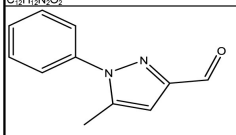
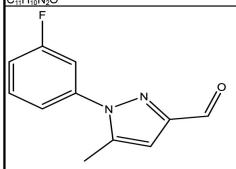
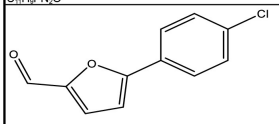
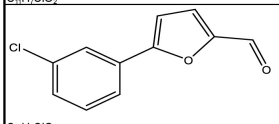
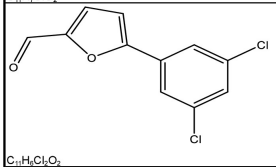
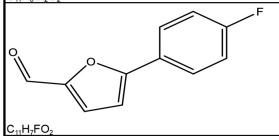


Figure 7. Synthesis of N-capped-C-capped FLIPs. Please click here to view a larger version of this figure.

STRUCTURE	ID	Exact poses	Exact poses in Top 50%	Closer poses (Pose number)	Closer poses in top 50%	PLP1	Consensus score (Pose number)	HB length	Remarks
 $C_{11}H_8ClN_2O$	5M_1	NA	NA	Top14(1,2,8,13,14,18,9,10,4,11,12,15,16,17)	10	53.38-53.31	4(1,2,8,13,14,18,9,10,4,11,12,15,16,17)	2.8	
 $C_{11}H_8ClN_2O$	5M_2	Top13,14(15,16)	NA	Top 4(10,7,9,8) Top 6-11(17,19,18,16,11,12)	9	Top4(57.29-57.17), Top6(50.97-47.4), Top13-14(41.09-41.08)	7(7,8,9,10,11,13)4(17,18)3(19),1(15,16)	2.5	
 $C_{11}H_8Cl_2N_2O$	5M_3	Top 10(18,19,20,13,16,5,12,3,15,17,7)	10	Top13-15(7,4,14)	NA	Top10(52.26-50.55)	3(18,13,5,15,17)2(19,20,16,12,3)	2.5(Top11)	All rest of the poses are closer
 $C_{12}H_{12}N_2O_2$	5M_4	NA	NA	Top15-19(19,15,16,17)	NA	Top15-19(45-42.81)	1(19), 0(15,16,17)	No HB with Trp217, Poses 15,16,17,18 form H bond with trp217	OCH3 group extends outside secondary pocket, Poses with both Hydrogen bonds interact better with the receptor
 $C_{12}H_{12}N_2O_2$	5M_5	NA	NA	Top4-10(10,11,13,15,14,7,8)	7	Top4-10(57.37-57.33)	7(10,11,13,15,14,7,8)	2.8(Top4-10)	OCH3 group is inside the secondary pocket
 $C_{11}H_{10}N_2O$	5M_6	NA	NA	Top12(15,19,5,6,7,18,2,10,8,16,9,14)	10	Top12(47.99-30.25)	6(19,18),3(15,2,16),4(6),2(5,7,8),1(10)	2.5	Only top 12 poses form both H-bonds, Closer poses, but substitution is required on Phenyl ring
 $C_{11}H_8FN_2O$	5M_7	NA	NA	Top5(19,13,14,18,20),Top7-11(4,3,5,2,7)	9	Top5(53.03-53.01)Top7-11(54.43-44.09)	6(13,14,18,19,20)5(3),4(4),2(5,7)	2.5, Ffor some poses 2.7	F- atom makes HB with Try225 in some poses
 $C_{11}H_7ClO_2$	5M_8	NA	NA	All the poses are similar	10	50.52-50.12	2	2.9 for H-bond with Trp217	
 $C_{11}H_7ClO_2$	5M_9	Top15-19(18,17,19,20)	NA	Top4-13(8,10,7,9,12,14,15,11,16,17)	7	Top4-13(56.79-54.12)Top15-19(42.99-41.26)	2(18,17,19,20),5(8,10,7,19,12,14,15,11,16,17)	2.5	Split H-Bond
 $C_{11}H_6Cl_2O_2$	5M_10	Top2-20(2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20)	9 poses	Top1 (1)	1	44.64-44.24	2(2,13),1(1,3,4,5,6,7,8,9,10,11,12,14,15,16,17,18,19,20)	2.5	Split H-Bond, very similar to 3,5DCPT
 $C_{11}H_7FO_2$	5M_11	NA	NA	All the poses are similar		45.55-44.4	3(1,2,3,4,10)	H-bond 2.9-3.1	Split H-bond

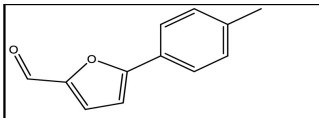
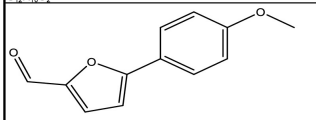
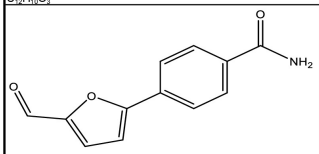
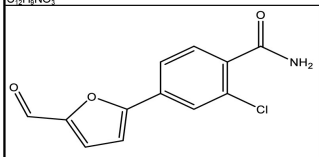
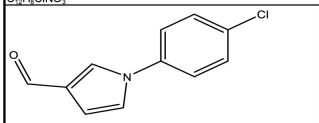
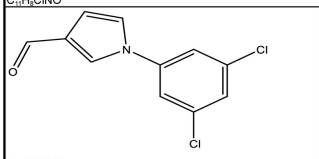
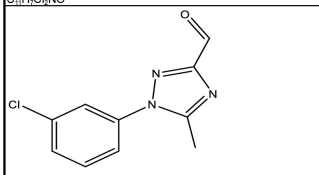
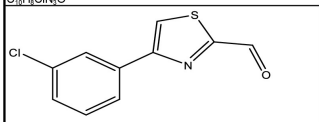
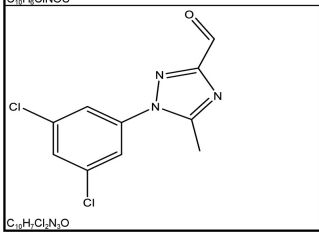
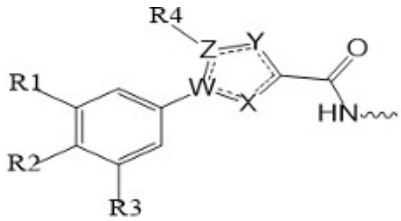
 $C_{12}H_{10}O_2$	5M_12	NA	NA	All the poses are similar	10	45.15-45.13	2(6,9,10),1(1,2,3,4,5,7,8,11,12,13,14,15,16,17,18,19,20)	2.9	Split H-bond
 $C_{12}H_{10}O_3$	5M_13	NA	NA	Top 1-13	10 poses	51.69-50.92	1,3	No H-bond with Trp217	OCH3 group extends outside secondary pocket
 $C_{12}H_9NO_3$	5M_14	NA	NA	Top12(10,14,15,9,2,3,14,19,1,4,5,7)	10	54.16-53.07	7(1-10)	3.1	OCH3 group extends outside secondary pocket
 $C_{12}H_8ClNO_3$	5M_15	NA	NA	Top16(2,3,5,7,8,10,12,13,9,16,17,15,18,14,20,19)	10	58.68-56.16	7	2.9	Amide group extends out side the secondary pocket, forms H-bond with Glu224
 $C_{11}H_8ClNO$	5M_16	Only one pose generated, does not superimpose well with native ligand	NA		NA		1		
 $C_{11}H_7Cl_2NO$	5M_17	No pose generated	NA		NA				
 $C_{10}H_6ClN_3O$	5M_18	NA	NA	Top9(1,4,9,11,5,7,8,6,10,13)	9	53.76-53.72	4	2.5	
 $C_{10}H_6ClNOS$	5M_19	NA	NA	Top 19 are closer poses	10	53.91-43.06	5,2,1,0	2.5	similar to 3,5-DCPT
 $C_{10}H_7Cl_2N_3O$	5M_20	Top3-9 (10,11,12,18,19,16,20)	7 poses	Top15,16,17(1,2,3)	NA	51.87-51.81	2	2.5	

Table 1. Library of small molecules docked and the resulting predictions of binding energy using the PLP1 scoring function.

Peptide	Purity	Column Dimensions	Method	Flow Rate	HPLC Retention Time	Theoretical MW	Observed MW
5843	>90%	4.6 × 250 mm	5-65% acetonitrile/ water/0.1% TFA	1 ml/min	23.9	732	732.6
5773	>90%	4.6 × 250 mm	5-65% acetonitrile/ water/0.1% TFA	1 ml/min	22.4	801.77	800.55

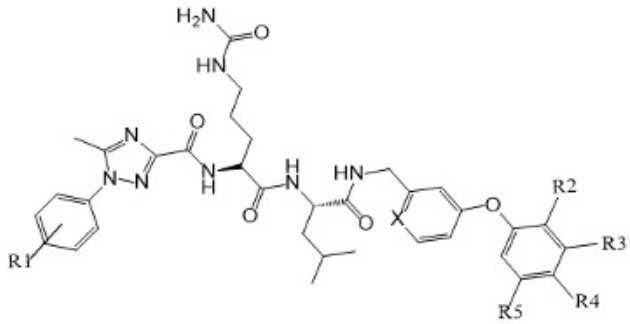
5774	>90%	4.6 × 250 mm	5-65% acetonitrile/ water/0.1% TFA	1 ml/min	24.2	767.33	766.5
5762	>90%	4.6 × 250 mm	5-65% acetonitrile/ water/0.1% TFA	1 ml/min	9.0	731.91	731.65
5763	>90%	4.6 × 250 mm	5-65% acetonitrile/ water/0.1% TFA	1 ml/min	11.2	800.8	799.5
5764	>90%	4.6 × 250 mm	5-65% acetonitrile/ water/0.1% TFA	1 ml/min	10.1	766.34	765.55
5771	>90%	4.6 × 250 mm	5-65% acetonitrile/ water/0.1% TFA	1 ml/min	9.4	749.9	749.6
5765	>90%	4.6 × 250 mm	5-65% acetonitrile/ water/0.1% TFA	1 ml/min	10.1	766.35	755.65
5766	>90%	4.6 × 250 mm	5-65% acetonitrile/ water/0.1% TFA	1 ml/min	9.4	761.9	761.55
5767	>90%	4.6 × 250 mm	5-65% acetonitrile/ water/0.1% TFA	1 ml/min	20.4	761.9	761.55
5776	>75%	4.6 × 250 mm	5-65% acetonitrile/ water/0.1% TFA	1 ml/min	23.3	785.7	784.55
5775	>90%	4.6 × 250 mm	5-65% acetonitrile/ water/0.1% TFA	1 ml/min	9.9	751.34	750.45

Table 2. Analytical Data for CGI Binding Peptides and FLIPS.



	SCCP ID	R1	R2	R3	R4	W	X	Y	Z	CDK2/cyclin A IC_{50} (μM)	CDK4/cyclin D1 IC_{50} (μM)
Triazole	5843	H	H	H	H	N	N	N	C	16.2±3	49.2 ±7.60
	5773	Cl	H	Cl	CH_3	N	N	N	C	4±0.6	27.3 ± 3.40
	5774	H	Cl	H	CH_3	N	N	N	C	11.5±3.3	12.0 ± 2.06
	5906	Cl	H	H	CH_3	N	N	N	C	5.7±0.99	26.5±7.49
	5907	CH_3	H	H	CH_3	N	N	N	C	13.8±1.13	51.05±16.05
Pyrazole	5762	H	H	H	CH_3	N	N	C	C	40.3±6.5	54.2 ± 3.05
	5763	Cl	H	Cl	CH_3	N	N	C	C	21.8±13.7	>100
	5764	Cl	H	H	CH_3	N	N	C	C	11.9±2.0	70.3 ±12.79
	5771	F	H	H	CH_3	N	N	C	C	29.6±12.2	65.9±6.60
	5765	H	Cl	H	CH_3	N	N	C	C	33.7±8.1	54.7 ±20.43
	5766	OCH_3	H	H	CH_3	N	N	C	C	64.1±4.2	>100
	5767	H	OCH_3	H	CH_3	N	N	C	C	>180	>180
Pyrrole	5776	H	Cl	H	H	N	C	C	C	>180	>180
	5775	Cl	H	Cl	H	N	C	C	C	>180	>180
Furan	5768	Cl	H	Cl	H	C	O	C	C	>180	>180
	5769	Cl	H	H	H	C	O	C	C	>180	>180
Imidazole	5760	H	H	H	CH_3	C	N	C	N	>180	>180
	5852	F	H	H	H	C	N	C	N	34.3±0.6	68.5 ±14.84
Thiazole	5583	H	Cl	H	H	C	N	C	S	>180	>180

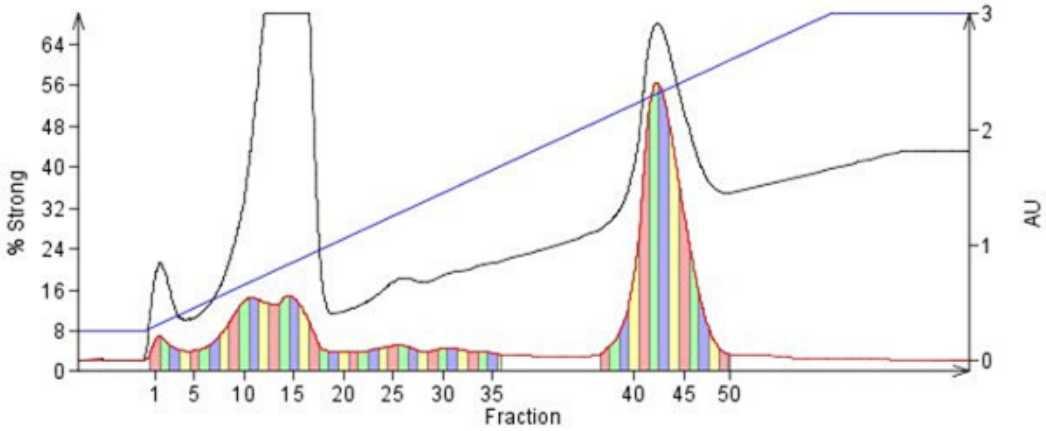
Table 3. *In vitro* binding of selected N-capped FLIPs to CDK2CA and CDK4CD.



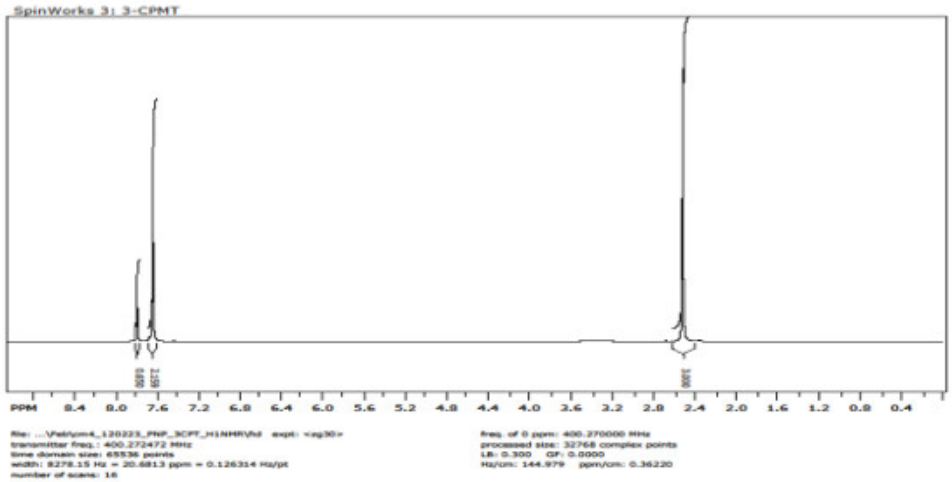
SCCP	R1	X	R2	R3	R4	R5	CDK2/cyclin A IC_{50} (μM)	CDK4/cyclin D1 IC_{50} (μM)
5807	4-chloro	C	H	H	H	H	106.1±26.2	>200
5824	4-chloro	N	H	F	H	H	53.2±11.6	>200
5823	4-chloro	N	H	H	F	H	18.1±4.0	>200
5822	4-chloro	N	H	H	Cl	H	54.4±0.9	>500
5825	4-chloro	N	CH_3	H	H	H	60±3.5	>500
5848	3,5-dichloro	N	H	Cl	H	H	61±10.8	120
5849	3,5-dichloro	C	H	Cl	H	Cl	>>180	120
5926	3,5-dichloro	C	H	F	F	H		

Table 4. ***In vitro*** **binding of selected N & C capped FLIPs to CDK2CA and CDK4CD.**

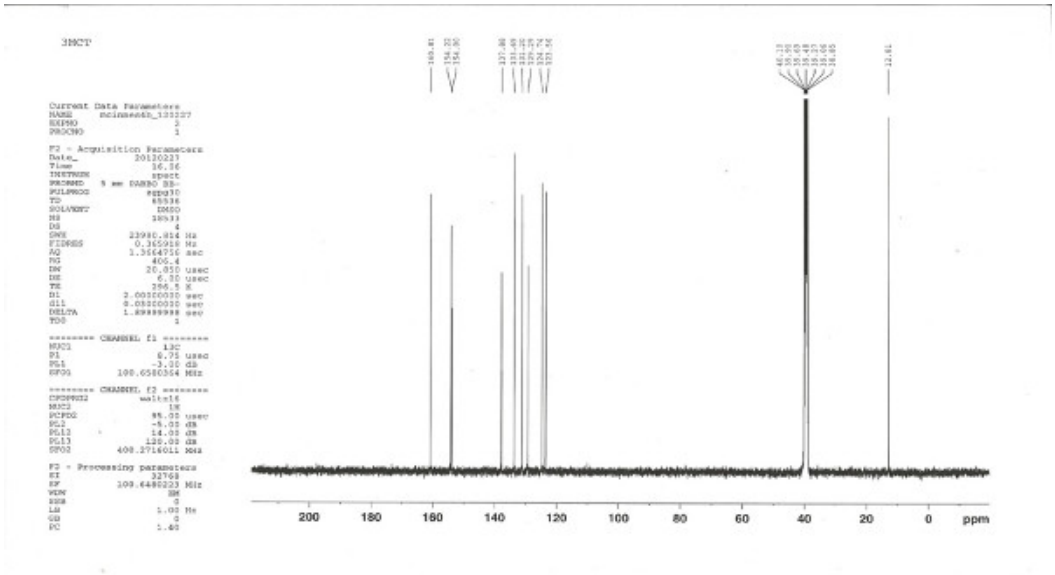
Supplementary Figure 1-5. Purification and characterization of 1-(3-chlorophenyl)-5-methyl-1H-1,2,4-triazole-3-carboxylic acid.



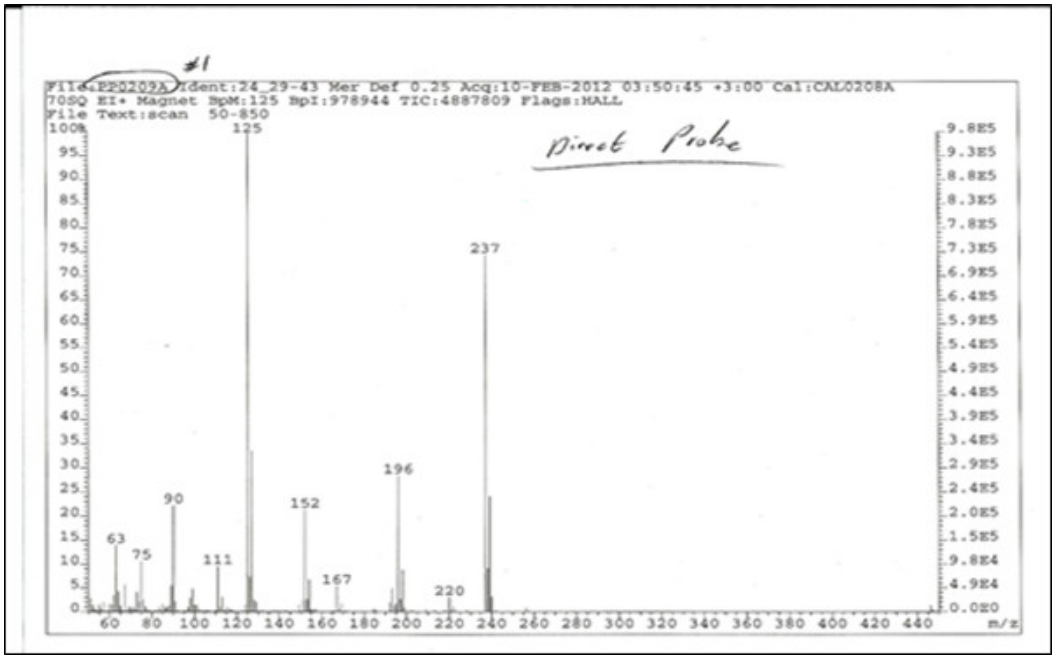
Supplementary Figure 1. Flash chromatogram of product obtained from step 1. There are four peaks eluting, the major peak eluting at 55% ethyl acetate / 45% hexanes was found to be the desired product. Please click here to view a larger version of this figure.



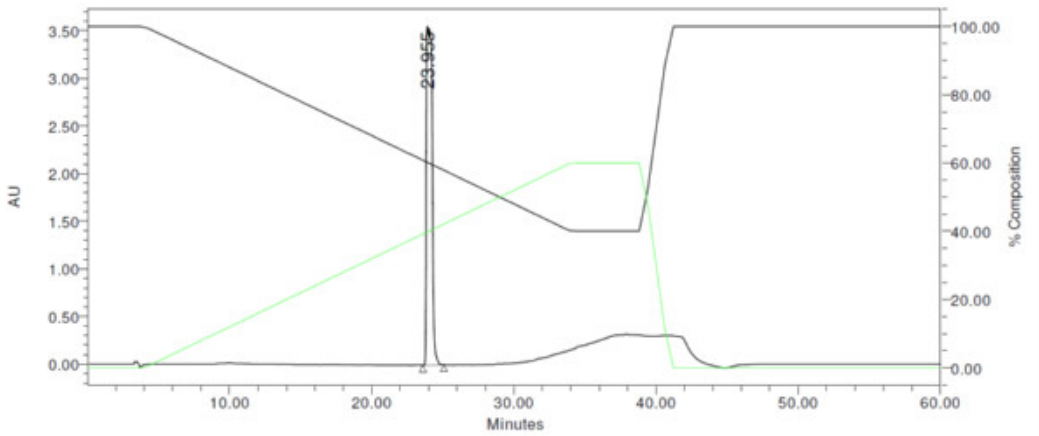
Supplementary Figure 2. ^{1}HNMR of 1-(3-chlorophenyl)-5-methyl-1H-1,2,4-triazole-3-carboxylic acid. The NMR spectrum shows 3 aliphatic methyl protons and 3 aromatic protons. The integration for all the three peaks is shown below the NMR spectrum. Please click here to view a larger version of this figure.



Supplementary Figure 3. ^{13}CNMR 1-(3-chlorophenyl)-5-methyl-1H-1,2,4-triazole-3-carboxylic acid. There are ten carbon atoms in the structure, ^{13}CNMR with 10 signals for each carbon atom. The integration for each peak is shown below the NMR spectrum. Please click here to view a larger version of this figure.



Supplementary Figure 4. MS of 1-(3-chlorophenyl)-5-methyl-1H-1,2,4-triazole-3-carboxylic acid. The molecular weight of the compound is shown as parent peak at 237. The actual molecular weight of the compound is 237.64. Please click here to view a larger version of this figure.



Processed Channel Descr.: PDA 226.0 nm

	Processed Channel Descr.	RT	Area	% Area	Height
1	PDA 226.0 nm	23.955	100077678	100.00	3537011

Supplementary Figure 5. HPLC of 1-(3-chlorophenyl)-5-methyl-1H-1,2,4-triazole-3-carboxylic acid. The purity of the product is determined as 100% as shown in the HPLC chromatogram. Please click here to view a larger version of this figure.

Discussion

Targeting protein-protein interactions (PPI) in drug discovery is highly challenging as these typically involve a large shallow contact interface comprised of numerous and diffuse contacts[19]. Furthermore, peptidic compounds which inhibit PPI's that are amenable to drug discovery are problematic due to their higher molecular mass, metabolic instability and poor bioavailability[20]. Current strategies that have been applied for the development of PPI inhibitors include design of proteomimetics and fragment based design. Examples of proteomimetics include stapled peptides, porphyrin scaffolds and cyclic α-helical mimetics although these are not without disadvantages[21,22]. Porphyrins (heterocyclic macrocycles comprised of pyrrole rings) are high molecular weight structures and are very hydrophobic[23]. Stapled peptides can be complex to synthesize and their mechanism of cell permeability is currently not fully known[6]. Conventional fragment based approaches require a highly sensitive detection method for testing weakly binding compounds and which also must be highly soluble. REPLACE is an iterative strategy comprised of computational prediction, synthetic organic chemistry and biological evaluation in order to convert peptidic inhibitors into FLIPs and ultimately non-peptidic drug-like compounds. Using REPLACE, fragment alternatives are designed and identified to mimic the interactions of binding determinants of a peptidic inhibitor. The combination of structural analysis along with synthetic methodologies and biological evaluation

results in the efficient design of molecules that interact with hot spots (sites of critical interaction) and complement the large contact surface area of the PPI binding site[1]. REPLACE overcomes the disadvantages of conventional fragment based design since the peptide acts as an affinity and solubility scaffold. Also the iterative nature of REPLACE means that the inhibitor is gradually converted in several steps therefore minimizing the possibility of complete potency loss through conformation change. PLAs are optimized in the context of the peptide prior to further truncation. In the case study we describe, REPLACE has been exploited to generate more drug-like inhibitors that selectively inhibit cell cycle CDK/cyclin complexes and possess significant anti-proliferative activity. Capped peptides have been identified as more drug-like mimics of the lead octapeptide. Key fragment alternatives discovered include N-caps based on (i) phenyl heterocyclic carboxylic acids[2], (ii) furan and thiazole carboxylic acids substituted with alkylamino groups and (iii) pyridine carboxylic acids combined with alkoxy or basic groups[14].

In this case study, the first stage of the REPLACE strategy was identification of fragment alternatives for key peptide determinants using high-throughput docking. LigandFit, a shape based docking method was employed for evaluating binding of fragments in the cyclin groove. LigandFit[15] generates poses that have high complementarity with a binding site through the use of Monte Carlo conformational search methods in conjunction with a shape based comparison filter. Use of a LigandFit protocol with the PLP1 energy grid and a minimization step to refine generated poses, was shown to reproduce known binding modes of previously determined N-capping groups. An accurate prediction of the binding affinity of the generated poses was provided by using the PLP1 scoring function which was shown to obtain the best results since it takes into account hydrogen bonding between the ligand and receptor. Specific H-bonds of CGI ligands with the cyclin groove have been shown to be important determinants of high affinity binding. Potential fragment alternatives for the N-terminus were synthesized or obtained from commercial sources and then used to generate FLIP molecules through coupling to an assembled peptide on a solid support. The ability of FLIPs to recapitulate the affinity of the native peptide is determined in a competitive binding assay using a known cyclin groove inhibitory peptide which is fluorescently labeled. After confirmation of the ability of FLIPs to bind with sufficient avidity to the CBG, the potency of the peptide-fragment hybrids can then be further refined through modifications to the Ncapping group. In addition, REPLACEments for C-terminus can explored for eventual combination with the optimized Ncaps. Further to this N terminal and C-terminal capping groups can be combined into a single more drug-like molecule[2]. Using this approach, compounds were obtained that have anti-proliferative activity in cancer cells lines that have deregulated CDK/cyclin/Rb/E2F1 pathways. Although REPLACE has been successfully applied, this strategy is not without limitations. These include the inaccuracy of any computational technique in predicting binding of docked molecules and synthetic issues in generating capping groups and FLIPs. Despite these, we have utilized REPLACE to generate more drug-like compounds that are cell permeable and have anti-proliferative activity. These compounds therefore have significant potential for further development as anti-tumor therapeutics.

Disclosures

The authors declare that they have no competing financial interests.

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