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Benzamide capped peptidomimetics as non-ATP competitive inhibitors of CDK2 using the REPLACE strategy

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ABSTRACT

Inhibition of cyclin dependent kinase 2 (CDK2) in complex with cyclin A in G1/S phase of the cell cycle has been shown to promote selective apoptosis of cancer cells through the E2F1 pathway. An alternative approach to catalytic inhibition is to target the substrate recruitment site also known as the cyclin binding groove (CBG) to generate selective non-ATP competitive inhibitors. The REPLACE strategy has been applied to identify fragment alternatives and substituted benzoic acid derivatives were evaluated as a promising scaffold to present appropriate functionality to mimic key peptide determinants. Fragment Ligated Inhibitory Peptides (FLIPs) are described which potently inhibit both CDK2/cyclin A and CDK4/cyclin D1 and have preliminary anti-tumor activity. A structural rationale for binding was obtained through molecular modeling further demonstrating their potential for further development as next generation non ATP competitive CDK inhibitors.

The cyclin dependent kinases (CDKs) are activated through binding to cyclin proteins which act as positive regulatory subunits. CDKs are known to play major roles in regulation of both checkpoint control of the cell cycle[1] and transcription initiation and elongation[2] where the latter effects are believed to contribute to toxicities observed with first generation CDK inhibitors evaluated in the clinic.[3,4] In addition to its roles in regulating the G1/S transition[5], CDK2/cyclin A (CDK2A) is intimately involved in progression through S phase.[6] A critical role of this latter complex is to phosphorylate the E2F1 transcription factor required for expression of S phase genes in a timely manner and to terminate its activity through inducing its dissociation from DNA.[7] Tumor cells, which intrinsically possess high levels of E2F1 transcriptional activity, can be sensitized to apoptosis through maintaining this protein in its DNA bound state as tumor cells exit S phase.[7] CDK2A inhibition would therefore push tumor cells past the apoptotic threshold and result in synthetic lethality with tumors that are null for Rb and/or p53 and therefore have deregulated E2F. Selective inhibition of cell cycle CDKs resulting in blocking of phosphorylation of the Rb protein and E2F1 can be attained through the cyclin groove substrate recruitment site and this protein–protein interaction can potentially be exploited therapeutically.[3,7–9] Non-ATP competitive inhibition through the cyclin groove also avoids competing with high intracellular concentrations of ATP. The previously optimized octapeptide inhibitor, HAKRRLIF based on a sequence found in the endogenous CDK inhibitor, $p21^{waf1}$ binds potently to the cyclin groove through multiple peptide determinants including ion pairing interactions of the KRR motif and through the hydrophobic side chains with primary and secondary lipophilic subsites present (Figs. 1 and 2).[10,11] Previous studies applying the REPLACE strategy[9,12–16] have identified capping groups replacing the N-terminal tetrapeptide that interact with either the secondary hydrophobic pocket or the critical arginine binding site but not effectively with both.[12–14,16] From this perspective, an alternative scaffold for presenting binding groups that could interact with both regions was sought and a substituted benzoic acid derivatives were generated and appended to a drug-like peptidomimetic sequence previously identified. The resulting fragment-peptide hybrids were found to potently inhibit CDK2/cyclin A and CDK4/cyclin D1 further validating the REPLACE strategy and showing promise as compounds with preliminary anti-proliferative activity useful for development as cell cycle CDK specific therapeutics.

Synthesis of benzoic acid derived N-terminal capping groups: Furoic acid capped FLIPs containing basic functional groups designed to interact with the arginine binding site were studied previously and shown to possess moderate binding and inhibition of CDK2/cyclin A.[13] Phenyl-(1,2,4)-triazole derived capping groups

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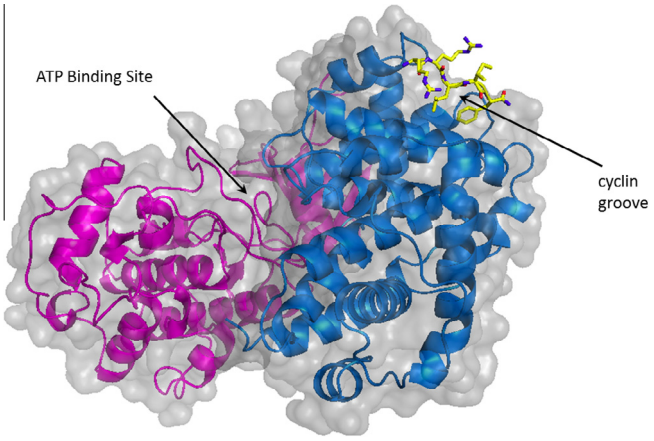


Figure 1. The cyclin groove is located on the cyclin regulatory subunit (blue secondary structure) of the CDK2 (magenta) complex and is required for substrate recruitment prior to phosphorylation. Peptidomimetics (shown in yellow) targeting this site inhibit CDK activity in a non-ATP competitive fashion.

successfully interface with the hydrophobic subsite however do not make effective contacts with the arginine binding site[12,14] and benzoic acid derived cyclin groove inhibitors do not interact with the minor hydrophobic pocket.[17] Based on these precedents, the design and synthesis of a unique series of benzamide fragment alternatives was undertaken (Table 1). Since this scaffold has considerable potential to further exploit peptide interacting residues and therefore more completely mimic the peptide-cyclin interface compared to previously investigated N-terminal capping groups[12–14], expansion of this as a core structure was investigated through additional substitutions. In the first instance, a number of piperazine containing functional groups (BA1 scaffold, Table 1, Supplementary information, SI) were installed at the 4 position by reductive amination of 4-formyl methyl benzoate with the appropriate cyclic amine to form the secondary amine. Capping groups including a derivatized piperazine group were installed using reductive amination subsequent to either methyl or benzyl ester protection and followed by hydrogenation or base hydrolysis as required (capping groups **2a**–**14a**, Table 1, Scheme 1).

To further functionalize the BA1 scaffold, analogs were incorporated where the piperazine ring was alkylated at the 3 and/or 4 position (Scheme 1, SI), To achieve this, reductive amination of 4-formylmethyl benzoate (with e.g., *tert*-butyl (*S*)-3-methylpiperazine-1-carboxylate was undertaken)[18] and alkylation of the free piperazine nitrogen where required was completed by reductive amination with the requisite aliphatic aldehyde to generate the tertiary amine prior to base hydrolysis[19] to liberate the capping group.

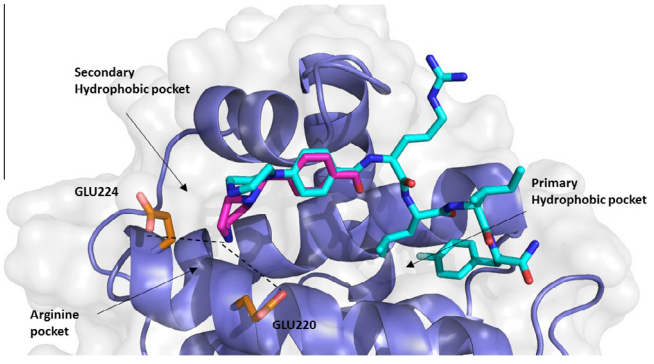


Figure 2. Calculated binding modes for the capping groups of compounds **2** and **3** with cyclin A (pdb id 2UUE represented as a Connolly surface and as ribbon diagram), showing an overlay of the 4-piperazinylbenzamide (magenta carbon atoms) and 4-piperazinylmethylbenzamide groups (cyan carbon atoms), respectively. As highlighted the methylene spacer in compound 3 results in more efficient ion pairing with Glu220 and 224 therefore leading to the observed potency increase.

The BA2 scaffold (Capping groups **15a**, **16a**, Table 1, Scheme 2, SI) was investigated to determine the consequences of restraining the rotation of the cyclic amine and the effect of this in interacting with the arginine binding site. Piperidin-4-ylidenemethyl benzoic acid derivatives were synthesized through Wittig reaction of methyl 4-((diethoxyphosphoryl) methyl)benzoate with amino piperidone (Scheme 2). The utility of this reaction and the potential SAR information could be further extended by high pressure hydrogenation of the ylidene to reduce the double bond and obtain the BA1 scaffold where X is CH_2[20] (Scheme 2, Table 1).

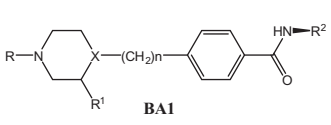
The BA3 scaffold (capping groups **17a**–**19a**, Table 1, Scheme 3, SI) was generated to determine the consequences of adding an amine spacing bridge (between the cyclic amine and the benzoic acid core) on the ion-pairing interactions with the arginine binding site. This scaffold also allowed significant diversity to be incorporated since it allowed not only different piperidine substitutions to be added but also enabled functionalization of the spacing nitrogen. These compounds were synthesized (Scheme 3) by reductive amination using the requisite amine followed by Boc-protection as necessary. The synthesis of analogs bis substituted at the 3 and 4 positions of the BA1 scaffold (Capping groups **20a**–**24a**, Table 2, Scheme 4, SI) was completed in a similar fashion to the 4-(piperazin-1-ylmethyl)benzoic acid derivatives however using reductive amination[18] of 4-formyl-3-hydroxybenzoic acid and the requisite amine. These were generated as described above to simultaneously interact with both the arginine binding site and the minor hydrophobic pocket. As required, the intermediate (i.e., 4-((4-(*tert*-butoxycarbonyl)piperazin-1-yl)methyl)-3-hydroxybenzoic acid) was alkylated to generate the 3-ethoxy and 3-propoxy derivatives.

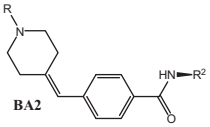
Further bis substituted analogs generated to probe the SAR of the two subsites were made by applying the Mitsunobu reaction[21] (capping groups **25a**–**28a**, Table 2, Scheme 5, SI) and included 4-piperidinyloxy, 3-methoxy-4-piperidinyloxy and 3,5-bis piperidinyloxy derivatives.

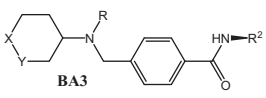
Structure activity relationship of peptides capped with mono-substituted benzoic acid derivatives: After generation of the capping groups, FLIP molecules (Table 1) were synthesized using previously described methods[12] (also see SI) and evaluated in a fluorescence polarization competitive binding assay shown to be a robust measurement of the ability of compounds to bind to the cyclin groove[12] (also see SI) and therefore inhibit CDK activity through blocking of substrate recruitment. Compounds containing the benzamide scaffold (Table 1) were potent mimics of the N-terminal tetrapeptide including **1**, possessing a guanidinomethyl group at the 4-position which was the most effective inhibitor (CDK2/cyclin A 0.69 μM and CDK4/cyclin D1—15.32 μM). Incorporation of the piperazine-1yl group (BA1, **2**, 35.16 μM) was a less effective substitution at the 4-position whereas addition of a methylene spacer into the Ncap (4-(piperazine-1ylmethyl), **3**) resulted in a 7-fold increase in binding to CDK2A (IC_{50} = 5.3 μM) and 2 fold increase in IC_{50} for CDK4D (12.87 μM). Varying the N-terminal tetrapeptide sequence in the 4-piperazinylmethylbenzamide context was undertaken.

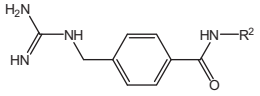
An optimized sequence (RLNpfF, **3**, 5.3 μM) was found to be 2 fold more potent than the native p21 sequence (RLIF, **4**, 12.9 μM) for cyclin A whereas replacement of the second arginine (ALIF, **5**) resulted in an inactive FLIP in comparison. Further derivatization of the core scaffold (BA1, Table 1) was undertaken and included the synthesis of analogs with varying substitution of the free piperazine nitrogen and addition of methyl groups onto the 2-position. Methylation of the piperazine N in compound **6** resulted in a 2 fold

Table 1
Structure–activity relationship of monosubstituted benzoic acid derived capped peptides

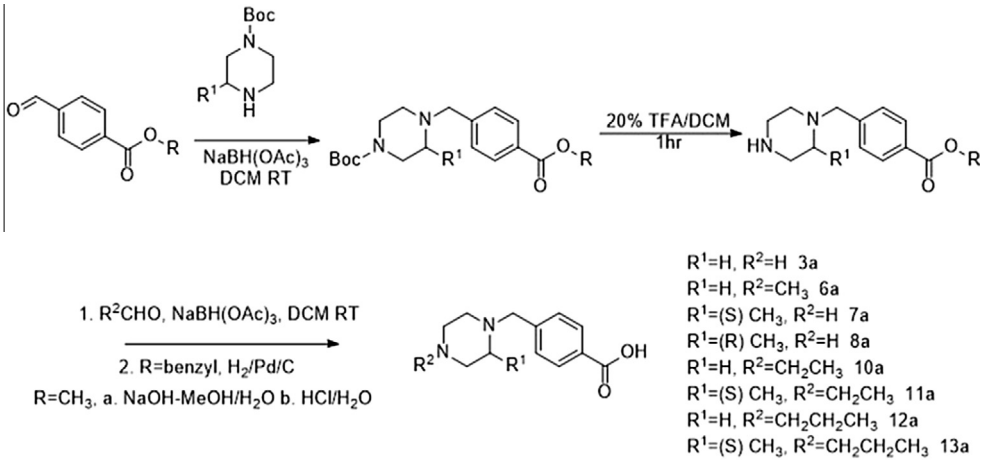






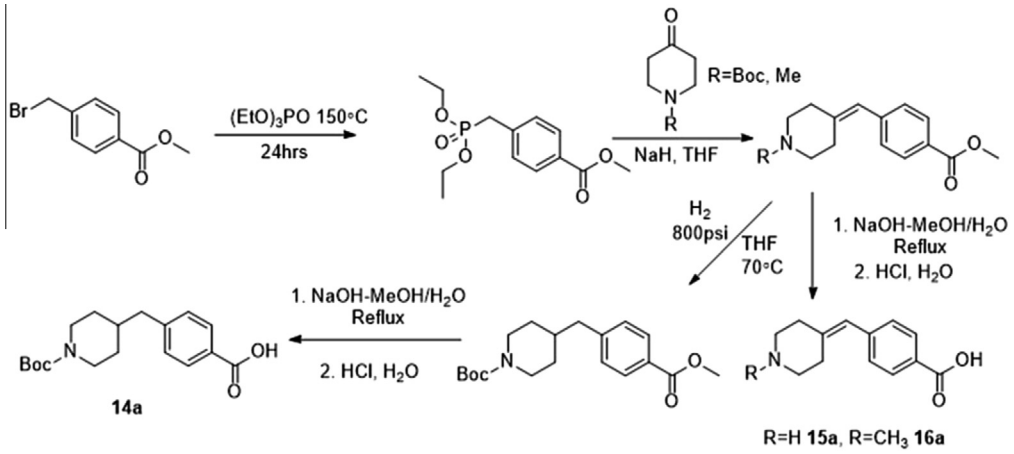


Compound	Capping group	Scaffold	*n*	X	R	R^1	R^2	CDK2/cyclin A IC_{50} (μM)	CDK4/cyclin D1 IC_{50} (μM)
1	1	540	—	—	—	—	RLNpfF	0.69 ± 0.34	15.32 ± 5.09
2	2a	BA1	0	N	H	H	Arg-Leu-Ile-Phe	35.16 ± 10.17	23.82
3	3a	BA1	1	N	H	H	RLNpfF	5.30 ± 0.99	12.87 ± 1.67
4	3a	BA1	1	N	H	H	Arg-Leu-Ile-Phe	12.89 ± 2.99	11.70 ± 5.92
5	3a	BA1	1	N	H	H	Ala-Leu-Ile-Phe	>100	51.49 ± 21.34
6	6a	BA1	1	N	CH_3	H	Arg-Leu-Ile-Phe	7.92 ± 1.39	11.31 ± 1.48
7	7a	BA1	1	N	H	(*S*) CH_3	Arg-βLeu-N-MePhe-NH_2	13.39 ± 4.35	>100
8	8a	BA1	1	N	H	(*R*) CH_3	Arg-βLeu-N-MePhe-NH_2	22.28 ± 5.22	45.45 ± 0.47
9	9a	BA1	1	N	N-methylpiperidine	H	Arg-βLeu-N-MePhe-NH_2	8.32 ± 0.36	48.89 ± 40.36
10	10a	BA1	1	N	CH_3CH_2	H	Arg-βLeu-3TA-NH_2	17.44 ± 13.39	74.28 ± 4.45
11	11a	BA1	1	N	CH_3CH_2	(*S*) CH_3	Arg-βLeu-3TA-NH_2	6.78 ± 2.72	45.60 ± 13.69
12	12a	BA1	-	N	$CH_3CH_2CH_2$	H	Arg-βLeu-3TA-NH_2	31.34 ± 15.71	8.52 ± 4.43
13	13a	BA1	1	N	$CH_3CH_2CH_2$	(*S*) CH_3	Arg-βLeu-3TA-NH_2	22.42 ± 9.66	9.36 ± 6.56
14	14a	BA1	1	CH_2	H	H	Arg-βLeu-3TA-NH_2	>30	—
15	15a	BA2	1	—	H	—	Arg-βLeu-3TA-NH_2	9.16 ± 8.74	>100
16	16a	BA2	1	—	CH_3	—	Arg-βLeu-3TA-NH_2	26.02 ± 7.13	69.91 ± 7.07
17	17a	BA3, X = CH_2, Y = N	1	—	CH_3	—	Arg-βLeu-3TA-NH_2	31	—
18	18a	BA3, X = N, Y = CH_2	1	—	H	—	Arg-βLeu-3TA-NH_2	>100	—
19	19a	BA3, X = N, Y = CH_2	1	—	CH_3	—	Arg-βLeu-3TA-NH_2	>50	—

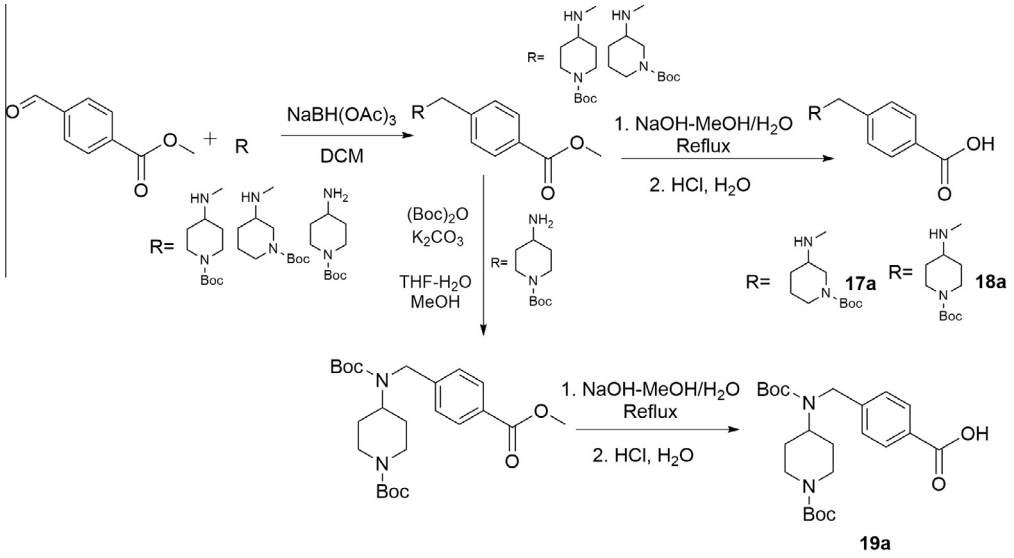


Scheme 1. Synthesis of BA1 Scaffold N-terminal capping groups.

increase in binding to CDK2A however did not affect interaction with CDK4D. Subsequent capping group modifications were investigated in either the Arg-βhomoLeu-N-MePhe-NH_2 or Arg-βhomoLeu-3-thienylalanine(3TA)-NH_2 contexts previously utilized to improve proteolytic stability and cellular permeability.[12] These sequences have comparable potency in vitro binding as the native p21 sequence, (RRLIF) to cyclin A even with one less amino acid residue. The extra methylene of the betahomoleucine residue mimics the Ile spacer residue in the p21 context, allows more efficient adaptation of the Leu and Phe and hence greater complementarity with the hydrophobic pocket. Addition of a methyl group onto the 2-position of the piperazine ring in the S-configuration resulted in similar CDK2A potency to the parent BA1 scaffold containing FLIP (**7**, IC_{50} = 13.4 μM) however the R-isomer was less active on CDK2A (**8**, IC_{50} = 22.3 μM). A further series of N-substituted piperazine derivatives were evaluated and included the placement of a piperidinyl group on the 4-N. This analog had improved activity relative to the free piperazine with just under a 2-fold increase (**9**, IC_{50} = 8.3 μM) in binding to CDK2A. Substitution of the same position with alkyl chains of increasing length revealed that both N-ethyl (**10**, IC_{50} = 17.4 μM) and N-propyl (**12**, IC_{50} = 31.3 μM) substitutions were deleterious to binding in the



Scheme 2. Synthesis of BA1 and BA2 Scaffold N-terminal capping groups.



Scheme 3. Synthesis of BA3 Scaffold N-terminal capping groups.

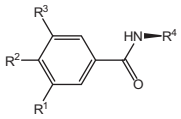
CDK2A context. The N-propyl containing FLIP possessed substantially improved binding to cyclin D relative to the N ethyl (IC_{50} = 45.6 vs 8.5 μM). The structure–activity of this series was extended by incorporating the 2S-methyl group in both the N-ethyl (**11**, IC_{50} = 6.8 μM) and N-propyl (**13**, IC_{50} = 22.4 μM) contexts. Each of these analogs were more potent than their unsubstituted counterparts when considering their CDK2/A activity with compound **11** attaining low micromolar binding and showing more than 2-fold greater binding.

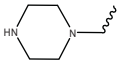
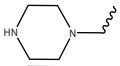
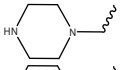
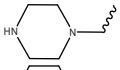
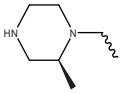
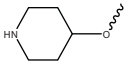
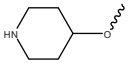
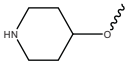
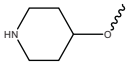
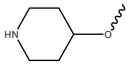
As mentioned above, FLIPS were generated containing the BA2 scaffold to determine the consequence of restraining the cyclic amine relative to the benzamide core structure. The 4-(piperidin-4-ylidenemethyl)benzamide FLIPs were generated in the free piperidine (**15**, IC_{50} = 9.2 μM) and N-methyl contexts (**16**, IC_{50} = 26.0 μM) with the results indicating that the order of potency was reversed from the BA1 scaffold. The BA3 scaffold incorporated both the 4-((piperidin-4-yl)amino)methyl) and 4-((piperidin-3-yl)amino)methyl)benzoic acid core structures (Table 1) to probe the spacial requirements of interaction of the ion pairing of the cyclic amine with the arginine binding site. In vitro testing of this series (**17**–**19**) showed that only weak binding to cyclin A was observed although the best analog had preliminary anti-proliferative activity.

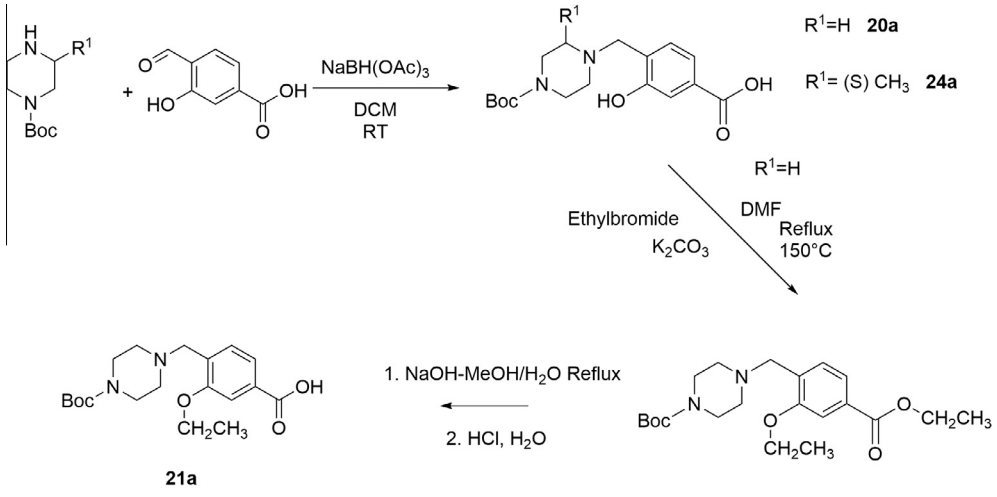
Structure activity relationship of peptides capped with 3,4-substituted benzoic acid derivatives: A further series of FLIPs incorporated bis substituted benzoic acid core structures in order to provide greater functionalization and more completely mimic the manifold interactions observed in the peptide/cyclin crystal structures.[10,11] This series includes substituents appended in both the piperazinylmethyl and piperidinyloxy contexts (Table 2) where introduction of substituents at the 3-position can potentially interact with this pocket and improve potency through additional van der Waals interactions.

The contribution of the 3-substituent was examined in the first instance through addition of a hydroxyl group at this position (**20**, IC_{50} = 4.56 μM) and was shown through testing to result in a 2 fold potency enhancement compared to **4** for CDK2A but without a concomitant increase in activity towards CDK4D (IC_{50} = 10.41 μM). Incorporation of a 3-ethoxy substituent in combination with the 4-piperazinylmethyl group (**21**, IC_{50} = 5.9 μM) lead to similar binding (compared to **20**) for binding to cyclin A and 2 fold better activity with respect to cyclin D (5.1 μM vs 10.4 μM). Comparable results are seen for **22** (RβLNMeF-NH_2) versus **20** (RLIF) in both cyclins where the N-cap is 3-hydroxy-4-(piperazin-1-ylmethyl) benzamide. The RβLNMeF counterpart (**23**) to the 3-ethoxy, 4-(piperazin-1yl)benzamide RLIF (**21**) has diminished potency for

Table 2
Structure–activity relationship of 3,4-substituted benzoic acid derived capped peptides



Compound	Capping group	R^1	R^2	R^3	R^4	CDK2/cyclin A IC50 (μM)	CDK4/cyclin D1 IC50 (μM)
20	20a	OH		H	Arg-Leu-Ile-Phe	4.56 ± 0.74	10.41 ± 0.61
21	21a	OCH_2CH_3		H	Arg-Leu-Ile-Phe	5.95 ± 2.53	5.14 ± 1.47
22	20a	OH		H	Arg-βLeu-N-MePhe-NH_2	7.42 ± 1.69	11.76 ± 1.01
23	21a	OCH_2CH_3		H	Arg-βLeu-N-MePhe-NH_2	22.61 ± 1.89	18.64 ± 9.72
24	24a	OH		H	Arg-βLeu-3TA-NH_2	13.1 ± 2.1	–
25	25a	H		H	Arg-Leu-Ile-Phe	31.34 ± 15.71	8.52 ± 4.43
26	25a	H		H	Arg-βLeu-N-MePhe-NH_2	40.38 ± 17.76	30.99 ± 1.18
27	27a	OCH_3		H	Arg-Leu-Ile-Phe	22.42 ± 9.66	9.36 ± 6.56
28	28a		H		Arg-βLeu-3TA-NH_2	>30	–

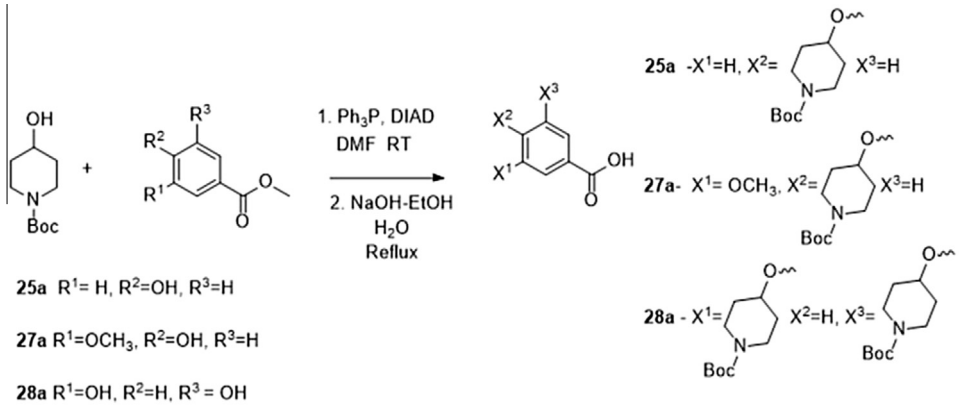


Scheme 4. Synthesis of bis substituted N-terminal capping groups based on BA1 Scaffold.

both cyclin A (22.61 μM) and D (18.64 μM). The 3,4 bis substituted version of FLIPs **7** (11.6 μM) and **9** (>100 μM) were generated where the 3-position was hydroxyl in both cases and where activity was retained in the context of the 2-methylpiperazine but not in the 4-((4-(1-methylpiperidin-4-yl)piperazin-1-yl)methyl)benzamide capped peptide.

As a means of generating further diversity, FLIPs containing piperidinyloxy substituents were synthesized in both mono and bis substituted contexts. Testing of the 4-piperidinyloxy FLIP (**25**, 8.52 μM) resulted in 4 fold more potent molecule than its piperazinyl counterpart (**2**, 23.82 μM) in binding to CDK4D (Tables 1 and 2) however was equipotent in binding to cyclin A in its CDK2 complex. FLIP **26** (IC_{50} = 31 μM) had a decreased binding to cyclin D relative to **25**. Bis substituted piperidinyloxy derivatives were generated (**27**, 22.4 μM and **28**, >30 μM) and compared to their mono 4-substituted counterparts. The 3-methoxy-4-piperidinyloxy derivative (**27**) was evaluated and demonstrated to have increased binding to CDK2A (22 μM vs 31 μM in cyclin A) and to be essentially equipotent towards CDK4D (9 μM vs 8 μM). The 3,4 bis piperidinyloxybenzamide capped peptide was generated and shown to have comparable activity to the analog mono-substituted at the 4 position.

Structural analysis of FLIP SAR with the cyclin A and D binding grooves: Previously described capping group scaffolds have led to potent CDK inhibitors however none have completely mimicked the interactions of the peptide binding groups. 1-(3,5-dichlorophe-



Scheme 5. Synthesis of piperidinyloxy benzoic acid N-terminal capping groups.

nyl)-5-methyl-1*H*-1,2,4-triazole-3-carboxylate derived capping groups primarily contact the secondary hydrophobic pocket and make H-bond interactions with Trp217 however bypass the Lys-Arg binding site.[12,14] Another series containing substituted heterocyclic core structures primarily bind to the acidic region but do not occupy the secondary pocket.[13] The benzoic acid scaffold was selected as a potentially effective core structure for presentation of multiple functional groups to interact more completely with the cyclin groove with the secondary pocket while also making ion-pairing interactions with acidic residues.

In order to improve the affinity of the N-capping group through the synthetic versatility of the benzoic acid scaffold, additional substituents were appended at the 4 position to include groups capable of ion-pairing with the cyclin groove. In the first instance, mono substituted benzoic acids with basic groups at the 3 or 4 positions were generated and included (guanidomethyl (**1**), piperazin1-yl (**2**), piperidin-4yloxy (**26**) and 4-methylpiperazin-1yl-methyl (**4**) (Table 1 and 2). The guanidinomethyl substitution was generated as a RLNpfF FLIP and the most potent cyclin groove inhibitor in this series (0.69 μM). The effectiveness of this compound is due to the very strong electrostatic interaction of the highly basic guanidine with the acidic region of cyclin A (E220, E224, Fig. 2).

This substitution was not selected for further optimization since it is likely that the presence of a guanidino group in addition to Arg5 would prevent cellular penetration. The increased activity of the 4-piperazinylmethylbenzamide FLIP (**4**) relative to its 4-piperazinyl counterpart (**2**) demonstrates that optimization of the ion-pairing interaction distance leads to greater binding (Table 1, Fig. 2). This was relevant in both the cyclin A and cyclin D1 contexts and structurally validated through the observation of closer salt bridge contacts in the modeled complexes with cyclin A (Fig. 2). For **2**, the ion pairing distances are 5.3 Å (GLU220) and 4.7 Å (GLU224) whereas in **3**, charge–charge contacts are significantly reduced to 3.3 Å and 3.8 Å. Compared to the Ncap with the free piperazine N4 nitrogen, the methylated analog (**6**) had increased activity in binding to cyclin A however similar affinity for cyclin D1. This probably results from a decreased desolvation penalty of the protonated and methylated amine and through increased van der Waals interactions (since methylation would not be expected to change the pK_a of this group substantially).

Modifications to the piperazine ring (**7**, **8**) included addition of R and S methyl group at the 2 position and resulted in almost 2-fold potency enhancement for the S methyl FLIP (relative to others containing the Arg-βLeu-N-MePhe-NH2 peptide sequence). The structural basis for this increase was suggested through molecular modeling in that the methyl group restricts rotation of the piperazine due to overlap with the phenyl ring and therefore contributes entropically to binding. Structural analysis indicates confirmation of the initial design hypothesis in that the piperazine 2-methyl group of **8** points out of the minor pocket however in **7**, the methyl group interacts effectively with the Trp217 resulting in tighter binding (Supplementary Fig. 1).

Further modifications included addition of alkyl substituents to the free piperazine nitrogen in both the unsubstituted and 2-methyl contexts. In both of these scenarios the N-ethyl substituent is more favorable for binding and therefore suggests that the propyl group sterically interferes with effective interaction with the pocket. This was further illustrated in the binding modes generated where interference with Glu220 is observed and therefore demonstrates that the ethyl group is optimal (data not shown). Investigation of the binding of FLIPs containing the BA2 scaffold (have an unsaturated link between the cyclic amine and the benzyl group) strongly suggests that this is beneficial as shown by comparison of **15** with the saturated version (**14**). Molecular modeling of these restrained analogs indicate that the double bond positions the basic nitrogen of the piperidine closer to both Glu220 and Glu224 and therefore is more optimal for ion pairing interactions (see Supplementary Fig. 2). Methylation of the piperidine N leads to a steric clash in this context providing an explanation for the decreased activity of **16**. Similar activity of the 3-aminopiperidine analogs (BA3, **17**) to **14** and the inactivity of the 4-aminopiperidine substitutions (BA3, **18**, **19**) were validated structurally by the suboptimal positioning of the cyclic amine (e.g., compared to **4**).

In an effort to build on the results obtained for the monosubstituted benzamide FLIPs, Ncapping groups were generated that combined substituents at the 3 and 4 positions to interact with the minor hydrophobic pocket and the arginine site. Addition of a phenolic OH group at the 3-position (FLIPs **20**, **22**, **24** vs **4**) in combination with the 4-piperazinylmethyl substituent leads to a 2–3 fold potency increase. While it was hypothesized that this group would increase potency through acting as an H-bond acceptor to the indole NH group of Trp217, molecular modeling results revealed that the binding mode is stabilized by an intramolecular H-bond while maintaining an effective salt bridge (Fig. 3). Alkylation of the OH to generate the ethoxy derivatives (**21**, **23**) yielded similar potency in terms of binding to cyclin A however a 2-fold increase was observed versus cyclin D1. The binding mode of these analogs suggests that the optimized cyclin D1 affinity results from greater complementarity with the secondary pocket in this context. The lack of potency increase for CDK2A compared to the free hydroxyl (**21** vs **20**) is suggested by modeling in that the intramolecular hydrogen bond is not possible in the alkylated context. Overall though, the activity profiles of these two compounds confirms the hypothesis that having a basic group at 4 position and an alkyl group at 3 position improves the activity of benzamido-FLIPs

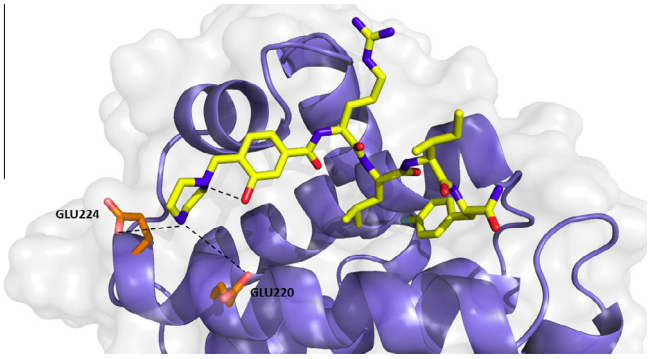


Figure 3. The calculated binding mode for compound **20** indicating the interactions of the piperazinyl group and the intramolecular H-bond stabilizing the conformation of the capping group.

through ion-pairing and van der Waals interactions. Based on this observation, FLIPs were generated with piperidinyloxy substituents at the 3 and/or 4 positions and in combination with a methoxy group. The 3-fold lower affinity of piperidin-4yloxy (**25**) monosubstituted analog for cyclin A was evidenced by suboptimal contacts with the key subsites. These interactions are more favorable in the cyclin D1 context thus correlating well with the enhanced binding of this analog. Compound **27** showed improved activity for cyclin A (vs **26**) and compares favorably with **21** which as described earlier effectively interacts with both the arginine and minor hydrophobic subsites on both cyclins. Inclusion of the piperidinyloxy group at both the 3 and 5 positions was detrimental to activity as these groups did not make effective ion pairing interactions with Glu220 and Asp283.

Previously identified N-terminal capping groups based on phenyltriazole substructures were used as a scaffold to generate a peptidomimetic that possessed greater drug-like properties and which demonstrated anti-proliferative activity in U2OS and DU145 cells.[12] The cellular activity (MTT assay) of selected benzamide capped FLIPS were assessed and compared with the previously reported compounds which were shown to have respectable growth inhibitory activity. For the most part evaluated compounds were inactive with the exception of FLIP **7** which exhibited weak but reproducible anti-proliferative activity (U2OS IC_{50} = 228.66 ± 56.5 μM, DU145 IC_{50} = 204.5 ± 34.64 μM). Another FLIP from the BA3 series was shown to have activity in the binding assay but also to have micromolar inhibitory activity in the MTT assay (compound **17**, U2OS IC_{50} = 153 ± 9 μM) demonstrating weak but consistent growth inhibition. Interestingly the two other FLIPS in the BA3 series synthesized were inactive in both the binding and cellular assays and therefore correlating well in this context. While the cellular activities are weak, they suggest that further optimization of potency and drug-like properties will enable facilitate promising compounds.

In conclusion, application of the REPLACE strategy to the CDK/cyclin groove in order to block substrate recruitment resulted in the elaboration of the 4-substituted benzamide scaffold as a replacement for the N-terminal tetrapeptide of the HAKRRIF, a sequence with optimized affinity. FLIPs incorporating derivatized 3,4 bis substituted benzamide groups were identified with low micromolar activity for both CDK2A and CDK4D with these compounds more completely mimicking the interactions of CGI peptides. When specific FLIPs incorporating these Ncaps were appended to a peptidomimetic sequence previously shown to impart drug-like properties, preliminary anti-proliferative activity was obtained. As a whole the results obtained indicate that this series has potential for further development as next generation CDK inhibitors based on non-ATP competitive blocking of kinase activity through the cyclin groove.

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Supplementary data

Supplementary data (characterization information for all peptides and FLIPS; additional figures, all synthetic procedures and characterization data and procedures used for the FP and cellular assays) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.05.067.

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