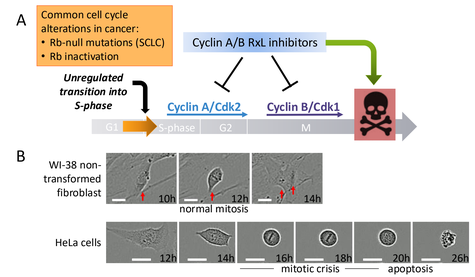
Permeable macrocyclic peptide Cyclin A/B RxL inhibitors: synthetic development, SAR and *in vivo* target validation

Nathan J. Dupper, Andrew T. Bockus, Siegfried S. Leung, Miguel P. Baldomero, Breena Walton, Justin A. Shapiro, Luis Hernandez, Bryan Lent, Megan DeMart, Joshua Luna, Catherine E. Gleason, Frances Hamkins Indik, Ranya Odeh, Meisam Nosrati, Daphne He, Ramesh Bambal, Peadar Cremin, Bernard Levin, Constantine Kreatsoulas, Rajinder Singh, James B. Aggen, Pablo D. Garcia

Introduction

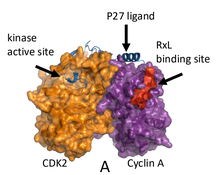
- Cyclins A and B (Cyclin A/B) bind and activate their cognate cyclin-dependent kinases (CDK) to regulate progression through S and G2/M phases of the cell cycle. These complexes orchestrate multiple activities during each phase of the cell cycle, the orderly transition between phases, and regulate critical cell cycle checkpoints. In addition, Cyclins bind certain critical substrates and regulators through the interaction of their RxL-motif with the Hydrophobic Patch (HP) on the Cyclin surface.
- The Inhibition of RxL-mediated substrate binding to Cyclin A has been postulated to be synthetically lethal in retinoblastoma (Rb) dysregulated cancers[1,2].
- While there are significant efforts to develop CDK inhibitors, only CDK4/6 inhibitors are in clinical use. Attempts to disrupt the protein-protein interactions between Cyclins and their substrates, such as E2F, at the HP have not advanced beyond early discovery previously.
- Here we will present on the synthesis of macrocyclic peptide Cyclin A and B RxL inhibitors, the SAR strategy throughout the program, highlights of key SAR, and initial *in vivo* efficacy results.

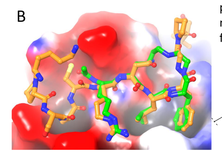
Figure 1. Model for synthetic lethality in Rb dysfunctional cells with Cyclin A/B RxL inhibitors

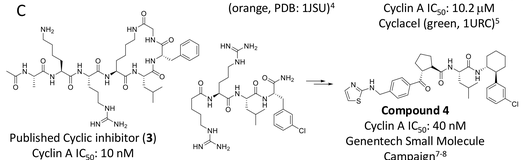


(A) Cells with dysregulated Rb pathway have unregulated transition into S-phase leading to stress in S and G2/M phases. Inhibition of Cyclin A/B RxL binding in these cancer cells triggers mitotic crisis and apoptosis. **(B) Live cell imaging in the presence of Cyclin A/B inhibitors:** WI-38 non-transformed fibroblast progress normally through mitosis, while HeLa cells undergo mitotic crisis and apoptosis. Incucyte time-lapse images captured at indicated times after addition of 3μM of an early Cyclin RxL inhibitor (bars = 50 μm).

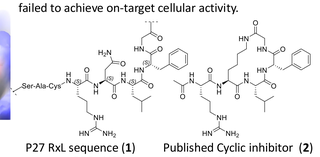
Figure 2. Cyclin A structure, ligand design and previous drug campaigns







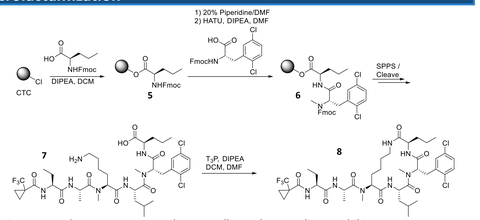
(A) Crystal structure of Cyclin A/CDK2 bound to p27. p27 (blue) binds to Cyclin A via its RxL motif (red) and localizes the phosphorylation substrate peptide of p27 within the active site of CDK2. **(B) Binding of RxL motif to Cyclin A.** Alignment of crystal structures of the p27 RxL-motif (Orange, PDB: 1JSU) and an early Cyclin A RxL inhibitor (green, 1URC) indicate that sidechain-to-tail cyclization can mimic the RxL binding motif. The sidechain-to-tail cyclization leads to "lariat3" type inhibitors that maintain the correct special orientation of the ligand, while allowing for a smaller peptide inhibitor. **(C) Previous attempts to drug Cyclin A.** Several programs aiming to develop Cyclin A RxL inhibitors relied heavily on a key arginine-glutamic acid interaction for potency. Attempts to remove this charged group were made, but the more "small molecule like" approach failed to achieve on-target cellular activity.



P27 RxL sequence (**1**)
(orange, PDB: 1JSU)[4]

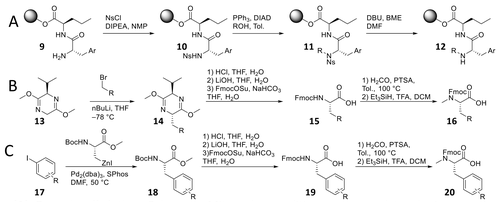
Published Cyclic inhibitor (**2**)
Cyclin A IC_{50}: 10.2 μM
Cyclacel (green, 1URC)[5]

Figure 3. Synthesis of Cyclin A inhibitors via SPPS and Macrolactamization



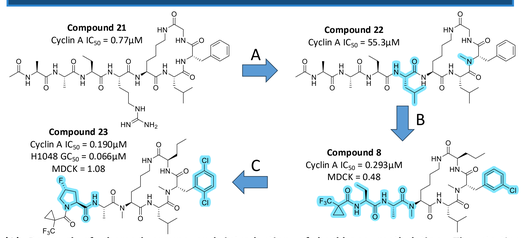
A Semi-automated process was used to rapidly explore Cyclin A inhibitor SAR. SPPS was conducted using established procedures on CTC resin. N-Alkylation of the backbone amides can be accomplished using on-resin Mitsunobu chemistry (see **Figure 4A**), or by the synthesis of the premethylated backbone (see **Figure 4B-C**). After cleavage from resin, the crude linear peptide was cyclized using T_3P.

Figure 4. Synthesis of N-alkyl and non-proteinogenic amino acids



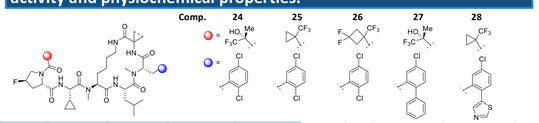
(A) On-resin alkylation. Fukuyama–Mitsunobu conditions were used to obtain on-resin N-alkylation of backbone amides. **(B) Synthesis of non-proteinogenic amino acids using Schöllkopf auxiliary.** At the onset of the SAR program, non-commercial amino acids were made with the Schöllkopf method. **(C) Synthesis of non-proteinogenic amino acids using Negishi coupling.** Once the phenylalanine position was identified as a major modulator of binding affinity, selectivity, and physicochemical properties, the cheaper, more efficient Negishi coupling was employed.

Figure 5. Development of initial cell active Cyclin A inhibitors



(A) Removal of charged groups and introduction of backbone methylation. The starting compound, from a Cyclacel publication[5], was first optimized for permeability. Removal of arginine and backbone methylation produced **Compound 22**. **(B) Path to sub-micromolar biochemical activity.** The initial decrease in activity was overcome by reducing compound size and optimization key lipophilic interactions. Additional tolerated N-methylations were also identified **(C) Path to cell active Cyclin A inhibitors.** After improving lipophilic interaction on the phenylalanine, and biasing the CF_3 toward a critical contact, an inhibitor with a sub-micromolar potency in in the NCI-H1048 cancer cell line was identified.

Figure 6. Identification of residues that modulated selectivity, cellular activity and physiochemical properties.

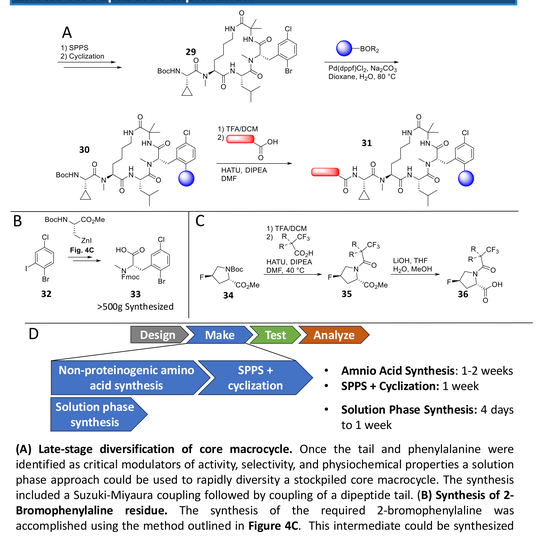


Comp.	Cyclin A* IC_{50} (μM)	Cyclin E IC_{50} (μM)	NCI-H1048 EC_{50} (μM)	MDCK (10^{-6}cm/s)
24	0.065	0.18	0.046	0.20
25	0.254	0.97	0.091	0.65
26	0.033	0.47	0.038	0.9
27	<0.020	0.22	<0.01	-
28	0.091	2.69	0.508	0.20

* Cyclin B Activity was consistently <0.020 μM

Several synthetic approaches were used to generate SAR around the molecule including the two positions shown in Fig. 6. Such modifications modulated multiple properties including biochemical potency and selectivity between different cyclins, cellular potency and permeability (see table). These and other studies allowed us to determine that dual cyclin A/B RxL inhibition is required for optimal synthetic lethality activity in E2F-driven cancers[9].

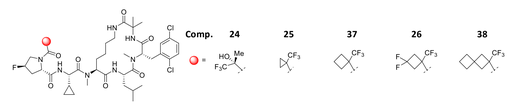
Figure 7. Solution phase derivatization using core macrocycle allows for rapid SAR exploration



- **Amnio Acid Synthesis**: 1-2 weeks
- **SPPS + Cyclization:** 1 week
- **Solution Phase Synthesis:** 4 days to 1 week

(A) Late-stage diversification of core macrocycle. Once the tail and phenylalanine were identified as critical modulators of activity, selectivity, and physiochemical properties a solution phase approach could be used to rapidly diversity a stockpiled core macrocycle. The synthesis included a Suzuki-Miyaura coupling followed by coupling of a dipeptide tail. (**B**) **Synthesis of 2-Bromophenylaline residue.** The synthesis of the required 2-bromophenylaline was accomplished using the method outlined in **Figure 4C**. This intermediate could be synthesized on a kilo scale. **(C) Synthesis of dipeptide.** The dipeptide used in the final coupling step could be accessed in a simple 3 step process (deprotection, coupling, saponification). **(D) Affect of late-stage diversification on the Design, Make, Test, Analyze cycle**. This approach greatly decreased the time required to synthesize compounds of this class.

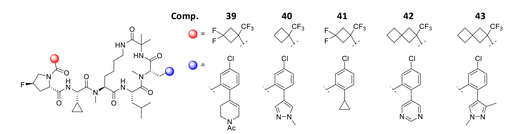
Figure 8. Optimization of N-terminus capping group



Comp.	Cyclin A* IC_{50} (μM)	Cyclin E IC_{50} (μM)	NCI-H1048 EC_{50} (μM)	MDCK (10^{-6}cm/s)	KSol	HLM CLint (μL/min/protein)
24	0.065	0.18	0.046	0.20	241.5	34.7
25	0.254	0.97	0.091	0.65	328.9	51.1
37	0.053	0.240	0.250	0.70	139.1	225
26	0.033	0.470	0.038	0.90	106.6	50.4
38	0.079	0.960	0.042	3.60	18.9	-

N-terminus capping groups were investigated for their affect on permeability, solubility and stability. Several stable and active capping groups were identified. * Cyclin B Activity was consistently <0.020 μM

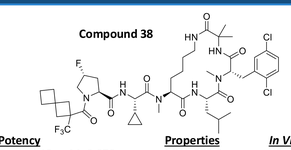
Figure 9. Combination of selectivity drivers at the N-terminus and 2-phenylalanine position



Comp.	Cyclin A* IC_{50} (μM)	Cyclin E IC_{50} (μM)	NCI-H1048 EC_{50} (μM)	MDCK (10^{-6}cm/s)	Ksol	DNAUC / Clp**
39	<0.020	2.57	0.096	-	-	-
40	<0.020	0.60	<0.010	0.3	223.8	0.39 / 42.8
41	0.026	0.38	<0.010	0.7	39.2	0.82 / 20.4
42	0.117	9.67	0.254	0.20	80.60	0.49 / 33.9
43	0.062	12.13	0.047	0.30	14.50	-

Selectivity driving substituents at the N-terminus and the 2-position of the Phenylalanine were combined. This effort produced numerous potent Cyclin A/B inhibitors with a range of selectivity profiles and *in vivo* DMPK behaviors. * Cyclin B Activity was consistently <0.020 μM. **Mouse, IV, 2 mg/kg.

Figure 10. Profile Compound 38: Potency, Properties, DMPK, and Kinome Scan.



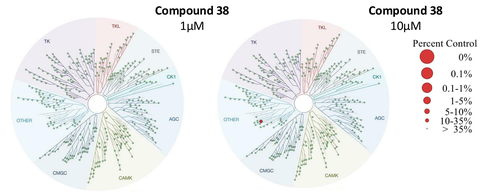
Potency
Cyclin A IC_{50} μM: 0.079
Cyclin B IC_{50} μM: <0.02
Cyclin E IC_{50} μM: 0.960
Cyclin A selectivity: 12.1
NCI-H1048[a] GI_{50} (μM): 0.042
NCI-H69[b] GI_{50} (μM): 0.016
OVCAR3[c] GI_{50} (μM): 0.059
WI-38 GI_{50} (μM): 9.27

a) NCI-H1048: Small-cell lung cancer
b) NCI-H69: Small-cell lung cancer
c) OVCAR3: Ovarian cancer

Properties
MW: 972.9
tPSA: 177.3
cLogP: 7.48
HBDs: 4

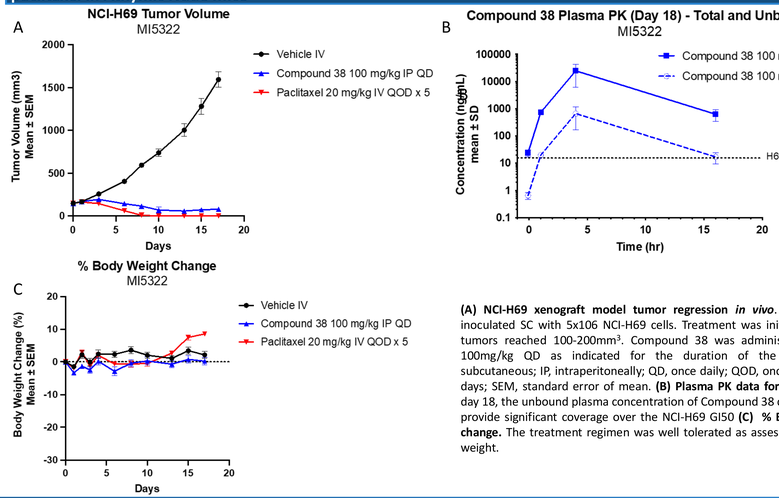
***In Vitro* DMPK**
KSol (μM): 18.94
MDCK P_{app}: 3.60
Caco-2 A-B P_{app}: 0.36
Caco-2 B-A P_{app}: 8.02
Efflux ratio: 22
Mouse PPB: 96.7%

%F (30 mg/kg PO):
4% alone
13.3% co-dosed w/ elacridar



KINOME*scan*™ (Eurofins Discovery) screening for off-target activity with Compound 38 at 1 and 10 μM. Kinases found to bind Compound 38 are marked with red circles—larger circles are associated with higher-affinity binding which indicates greater kinase inhibition. No significant inhibition of kinase activity was observed.

Figure 11. NCI-H69 small-cell lung cancer xenograft model data with Compound 38 in comparison to positive control paclitaxel in Athymic Nude mice



(A) NCI-H69 xenograft model tumor regression *in vivo*. Mice were inoculated SC with 5x106 NCI-H69 cells. Treatment was initiated when tumors reached 100-200mm^3. Compound 38 was administered IP at 100mg/kg QD as indicated for the duration of the study. SC, subcutaneous; IP, intraperitoneally; QD, once daily; QOD, once every two days; SEM, standard error of mean. **(B) Plasma PK data for day 18.** On day 18, the unbound plasma concentration of Compound 38 continued to provide significant coverage over the NCI-H69 GI50 **(C) % Body weight change.** The treatment regimen was well tolerated as assessed by body weight.

Conclusion

- Macrocyclic Cyclin A/B RxL inhibitors were optimized for permeability by the removal of polar sidechains, N-alkylation of the backbone amides and tuning of the hydrophobic interactions
- The early SAR campaign relied on semi-automated solid phase peptide synthesis making use of commercially available building blocks. Later in the SAR campaign our synthetic approach shifted to a blend of non-proteinogenic amino acid synthesis, SPPS, and late-stage functionalization.
- The phenylalanine and C-terminus capping group were identified as 2 key drivers of selectivity, cell potency, and physiochemical properties.
- This SAR campaign resulted in the discovery of peptide macrocycle **Compound 38**, A highly potent Cyclin A/B RxL inhibitor.
- **Compound 38** was used for *in vivo* target validation of this novel mechanism in an animal xenograft tumor models via IP administration.
- Selective inhibition of Cyclins offers a mechanistic mode of action distinct from inhibition of Cyclin dependent kinases. Given their compelling characteristics we are progressing development of orally bioavailable macrocyclic Cyclin A/B RxL inhibitors to clinical trials.

References

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