Orally bioavailable macrocycles that target Cyclins A and B RxL motifs cause tumor regression in xenograft models and *in vitro* show activity across multiple cancer types

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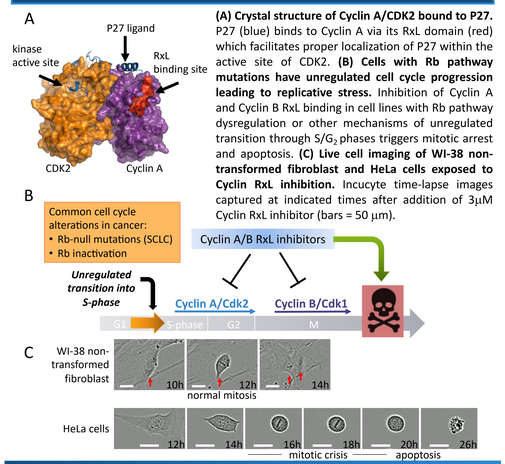
BACKGROUND

- Cyclins A and B are key cell cycle regulators that bind and activate their cyclin-dependent kinase (CDK) partners (Cyclin A/CDK1, Cyclin A/CDK2 and Cyclin B/CDK1) to regulate progression through S and G_2/M phases. Cyclins contain a highly conserved MRAIL motif forming the hydrophobic patch that facilitates interaction with a subset of Cyclin/CDK targets containing RxL (Cy) motifs (Figure 1A).
- While CDK kinase inhibitors are approved for clinical use, attempts to target protein-protein interactions between cyclins and their substrates, such as E2F, have so far not advanced beyond early research investigation[1].
- Inhibition of substrate binding to Cyclin A has been postulated to be synthetically lethal in retinoblastoma (Rb) mutated cancers[2].
- Using structure-guided design we have developed cell-permeable macrocycle compounds that inhibit the RxL-mediated binding of substrates to both Cyclin A/CDK2 and Cyclin B/CDK1 complexes (Cyclin A/B RxL inhibitors) and have demonstrated that synthetic lethality requires inhibition of both Cyclins A and B (Figure 1B & 1C).
- Here we present preclinical data supporting the development of an orally bioavailable therapeutic targeting Cyclins A and B.

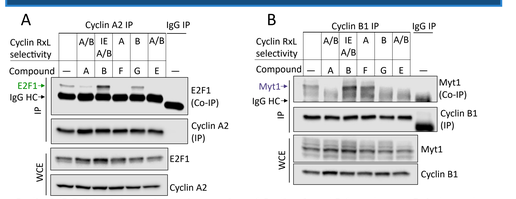
Figure 1. Model for synthetic lethality in Rb dysfunctional cells with Cyclin A/B RxL inhibitors



(A) Crystal structure of Cyclin A/CDK2 bound to P27. P27 (blue) binds to Cyclin A via its RxL domain (red) which facilitates proper localization of P27 within the active site of CDK2. **(B) Cells with Rb pathway mutations have unregulated cell cycle progression leading to replicative stress.** Inhibition of Cyclin A and Cyclin B RxL binding in cell lines with Rb pathway dysregulation or other mechanisms of unregulated transition through S/G_2 phases triggers mitotic arrest and apoptosis. **(C) Live cell imaging of WI-38 non-transformed fibroblast and HeLa cells exposed to Cyclin RxL inhibition.** Incucyte time-lapse images captured at indicated times after addition of 3μM Cyclin RxL inhibitor (bars = 50 μm).

RESULTS

Figure 2. Cyclin A/B RxL inhibitors displace RxL-mediated interactors for both Cyclins A and B



Cyclin A/B RxL inhibitors disrupt interaction of Cyclins A and B from known RxL-dependent binding partners. (A,B) NCI-H1048 cells were incubated with DMSO or the indicated compounds for 2h (compounds A, B, G and H, 300nM; compound E, 30nM). **(A) Cyclin A/B and Cyclin A RxL inhibitors disrupt the Cyclin A2:E2F1 interaction.** Binding of Cyclin A2 with E2F1 was determined by co-immunoprecipitation (IP) with anti-Cyclin A2 antibodies. Cyclin A2 IPs were analyzed by SDS-PAGE and Western Blot. **(B) Cyclin A/B and Cyclin B RxL inhibitors disrupt the Cyclin B1:Myt1 interaction.** Binding of Cyclin B1 with Myt1 was determined by co-immunoprecipitation (IP) with anti-Cyclin B1 antibodies. Cyclin B1 IPs were analyzed by SDS-PAGE and Western Blot.

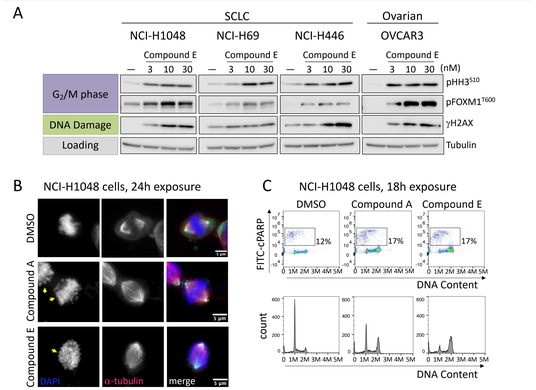
IE A/B, inactive enantiomer of compound A; IgG HC, immunoglobulin G heavy chain; IP, immunoprecipitation; WCE, whole cell extract

Table 1. Biochemical potency and cellular activity of Cyclin A/B RxL inhibitors

Compound name		A	E	B	F	G
RxL Inhibitor Selectivity		Cyclin A/B		Cyclin A/B IE[1]	Cyclin A	Cyclin B
Biochemical Potency FP IC_{50} (μM)[2]	Cyclin A	0.13	<0.02	>20	0.049	1.42
	Cyclin B	<0.02	<0.02	4.7	0.31	0.02
Cellular Potency Proliferation GI_{50} (μM)	NCI-H1048[3]	0.032	0.004	12.8	2.98	2.80
	NCI-H69[3]	0.013	0.002	>20	5.21	1.77
	NCI-H446[3]	0.065	0.009	NA	4.18	4.35
	OVCAR3[4]	0.064	0.01	>20	>20	6.44
	WI-38[5]	11	12	>20	>20	>20

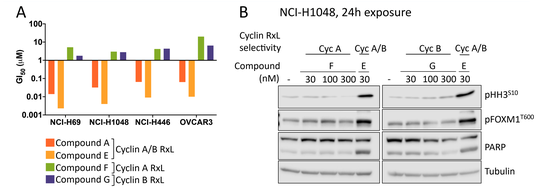
[1] Compound B is the inactive enantiomer (IE) of compound A , [2] Fluorescence polarization competitive assay (Cyclin A/CDK2; Cyclin B/CDK1), [3] small cell lung cancer (SCLC) cell line; 5-day proliferation MTT assay, [4] ovarian cancer cell line; 5-day proliferation MTT assay, [5]non-transformed fibroblast cell line; 3-day proliferation MTT assay

Figure 3. Cyclin A/B RxL inhibitors induce DNA damage, G_2/M phase arrest, mitotic abnormalities and apoptosis



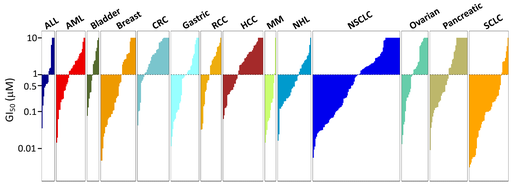
(A) Cyclin A/B RxL inhibitors induce markers of G_2/M accumulation and DNA damage. SCLC or ovarian cell lines were incubated with DMSO or compound E for 24h at the concentrations indicated. Cell lysates were analyzed by Western Blot. **(B) Uncompacted metaphase plates are observed in H1048 cells treated with Cyclin A/B RxL inhibitors.** NCI-H1048 cells were incubated with DMSO or 100nM compound A or E for 24h. Cells were then processed in an immunofluorescence assay to detect α-tubulin (red) and Centrin 3 (green) and co-stained with DAPI (blue) to detect DNA. Images were collected via widefield microscopy (63X objective). Arrowheads indicate uncompacted metaphase plates. **(C) Apoptosis is triggered 18h after exposure to Cyclin A/B RxL inhibitors.** NCI-H1048 cells were treated for 18h with DMSO or 30nM Compound A or E before collecting for flow cytometry. Induction of cleaved PARP (cPARP) was assessed by detection of FITC-cleaved PARP and DNA content (FxCycle). Top panels; cleaved PARP (box indicates cPARP positive gate with percent of single cells expressing cPARP indicated outside), Bottom panels; cell cycle profile.

Figure 4. Activity against both Cyclins A and B is required to induce accumulation of G_2/M phase markers and apoptosis



(A) Waterfall plot for SCLC cell lines (NCI-H69, NCI-H1048 and NCI-H446) and OVCAR3 ovarian cell line. Cells were plated in 96 well plates and exposed to compounds A, E, F or G for 5 days. Cell growth was determined by MTT assay. **(B) Cyclin A or Cyclin B selective RxL inhibitors do not induce markers of G_2/M accumulation or apoptosis.** NCI-H1048 cells were incubated with DMSO or compounds F, G or E for 24h at the concentrations indicated. Cell lysates were analyzed by Western Blot.

Figure 5. Cell lines from multiple indications exhibit sub-micromolar sensitivity to Compound A



GI_{50} waterfall plots in response to Compound A. A substantial number of cell lines from multiple indications including solid tumors and hematological malignancies respond (as assessed by GI_{50} < 1 μM) to inhibition of Cyclin A/B RxL binding. Cell lines were exposed to Compound A for 4-8 days depending on length of time required for at least two cell doublings to occur. Cell growth inhibition was determined by Cell Titer Glo assay.

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; RCC, renal cell carcinoma, HCC, hepatocellular carcinoma; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer

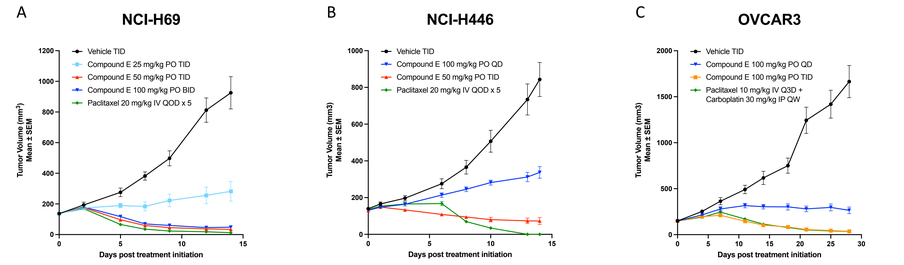
Figure 6 . Sensitivity of SCLC cell lines to Cyclin A/B RxL inhibition correlates with RB alteration and increased E2F1 target gene expression

(A) SCLC cell line sensitivity to Cyclin A/B RxL inhibition correlates significantly with increased gene expression for E2F targets and G_2/M checkpoint pathway. Gene expression heatmap of 42 SCLC cell lines for the selected Hallmark pathway scores. Each column represents a distinct SCLC cell line ordered from most sensitive to least sensitive based on Compound A GI_{50}. Color-coded panel on top depicts genomic alteration status of TP53 and RB1. The Gene Set Variation Analysis (GSVA) method[3] was utilized to identify Hallmark pathway scores (MSigDb Hallmark collection)[4] p value (*p <0.05) was calculated by Wilcoxon rank sum test. **(B) E2F1 expression level correlates with sensitivity in SCLC cell lines.** Baseline mRNA expression of E2F1 in SCLC cells was determined by RNAseq, ** p value was calculated by Fisher's exact test.

Figure 7. Cyclin A/B RxL inhibitors, exemplified by Compound E, exhibit no significant inhibition of kinase activity

KINOME*scan*™ (Eurofins Discovery) screening for off-target activity with Compound E at 10 μM. Active site-directed competition binding assay quantifies interactions between test compound and a panel of 468 human kinases and kinases with tumor driver mutations. Shown is a representation of the human kinome phylogenetic tree (TREEspot™ Interaction Maps). No kinases were found to bind Compound E—larger circles are associated with higher-affinity binding which indicates greater kinase inhibition. No inhibition of kinase activity was observed.

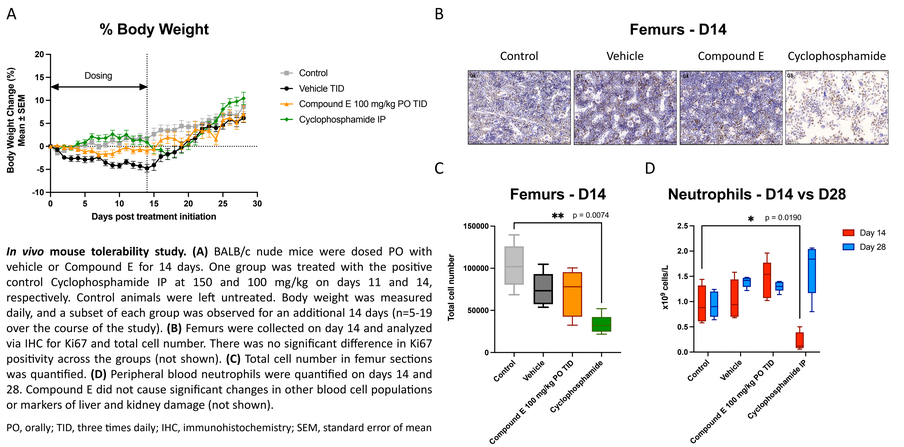
Figure 8. Oral dosing of a Cyclin A/B RxL inhibitor induces tumor regression in *in vivo* SCLC and ovarian cancer xenograft models



***In vivo* CDX efficacy studies. (A)** Mice were inoculated SC with $5x10^6$ NCI-H69 cells, **(B)** $5x10^6$ NCI-H446 cells, or **(C)** $2x10^7$ OVCAR3 cells. Treatment was initiated when tumors reached 100-200mm^3. Compound E was administered PO at 25 mg/kg TID, 50 mg/kg TID, or 100 mg/kg QD, BID, or TID as indicated for the duration of each study (n=10 for all groups). All treatment regimens were tolerated as assessed by body weight measurements (not shown).

SC, subcutaneous; PO, orally; QD, once daily; BID, twice daily, TID, three times daily; QOD, once every two days; Q3D, once every three days; QW, once weekly; SEM, standard error of mean

Figure 9. Efficacious dosing regimen of Cyclin A/B RxL inhibitor is well tolerated in mice and does not cause peripheral neutropenia or depletion of bone marrow cells



***In vivo* mouse tolerability study. (A)** BALB/c nude mice were dosed PO with vehicle or Compound E for 14 days. One group was treated with the positive control Cyclophosphamide IP at 150 and 100 mg/kg on days 11 and 14, respectively. Control animals were left untreated. Body weight was measured daily, and a subset of each group was observed for an additional 14 days (n=5-19 over the course of the study). **(B)** Femurs were collected on day 14 and analyzed via IHC for Ki67 and total cell number. There was no significant difference in Ki67 positivity across the groups (not shown). **(C)** Total cell number in femur sections was quantified. **(D)** Peripheral blood neutrophils were quantified on days 14 and 28. Compound E did not cause significant changes in other blood cell populations or markers of liver and kidney damage (not shown).

PO, orally; TID, three times daily; IHC, immunohistochemistry; SEM, standard error of mean

CONCLUSIONS

- Macrocyclic Cyclin A/B RxL inhibitors have been optimized for oral bioavailability and show single agent tumor regression in SCLC and ovarian xenograft models.
- At efficacious doses, Cyclin A/B RxL inhibitors are well tolerated with no neutropenia, decrease in body weight, or markers of liver and kidney damage observed.
- Cyclin A/B RxL inhibitors bind to Cyclin A/CDK2 and Cyclin B/CDK1 complexes with nanomolar biochemical potency and disrupt Cyclin A and B RxL-dependent interactions in cells. Consistent with a synthetic lethal mechanism, Cyclin A/B RxL inhibitors selectively block cancer cells at the G_2/M phase and induce apoptosis.
- Cyclin A/B RxL inhibitors show activity in cell lines from broad cancer indications and sensitivity correlates with increased expression of E2F1 target genes.
- Selective inhibition of Cyclins offers a mechanistically distinct mode of action from strategies that inhibit cell cycle kinases. Given their compelling characteristics we are progressing development of these macrocyclic Cyclin A/B RxL inhibitors to the clinic.

References

1. Mendoza et al. (2003) *Cancer Res.* 63, 1020–1024; 2. Chen et al. (1999) *PNAS* 96, 4325–4329; 3. Hänzelmann et al. (2013) *BMC Bioinformatics* 14:7, 1471-2105; 4. Liberzon et al. (2015) *Cell Syst* 1, 417-425