Macrocycles inhibiting RxL-mediated binding of substrates to Cyclin A are synthetic lethal in Rb mutated small cell lung carcinoma (SCLC)

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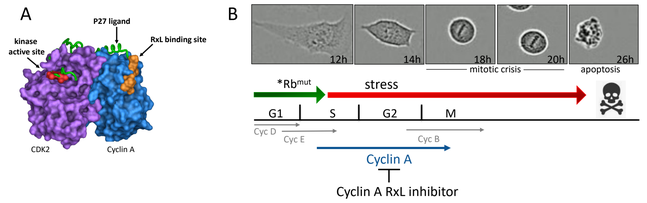
Background

- Cyclin A, a member of the cyclin family, when bound to either CDK2 or CDK1 regulates progression through S and G_2/M phases of the cell cycle. Cyclin A contains a highly conserved MRAIL motif that facilitates interaction with a subset of Cyclin A targets containing Cy (RxL) motifs (Figure 1A).
- Inhibition of substrate binding to Cyclin A has been postulated to be synthetically lethal in retinoblastoma (Rb) mutated cancers[1] (Figure 1B). Importantly, Rb mutations are found in ~90% of SCLCs[2].
- While cyclin-associated (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[3].
- Using structure-guided design we have developed cell-permeable macrocycle compounds that selectively inhibit the RxL-mediated binding of substrates to the Cyclin A/CDK complex.
- Novel macrocyclic inhibitors that disrupt Cyclin A RxL-mediated substrate binding have demonstrated G_2/M accumulation and apoptosis in Rb negative SCLC *in vitro* consistent with synthetic lethality, and anti-proliferative effects *in vitro* and *in vivo*.

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



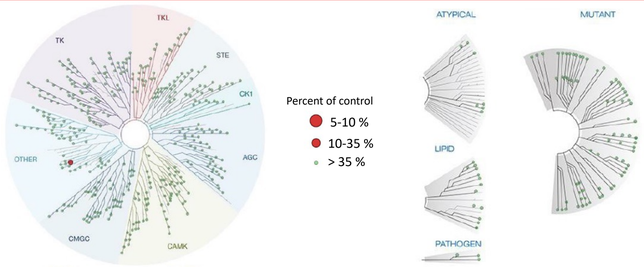
(A) Crystal structure of Cyclin A/CDK2 bound to p27. P27 (green) binds to Cyclin A via its RxL domain (orange) which facilitates proper localization of p27 within the active site of CDK2 (red). **(B) Cells with dysfunctional Rb have unregulated cell cycle progression leading to replicative stress.** Inhibition of Cyclin A RxL binding in Rb dysfunctional cell lines triggers mitotic arrest and apoptosis. Live cell imaging of HeLa cells (Incucyte, 20X) exposed to 3μM Cyclin RxL inhibitor. Pictures capture the same cell imaged after compound addition. Time (in hours) after compound addition is indicated.

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

Compound	Cyclin A/CDK2 Biochemical IC_{50} (μM)[1]	NCI-H1048 SCLC Proliferation GI_{50} (μM)[2]	WI-38 fibroblast Proliferation GI_{50} (μM)[3]
A	0.14	0.043	11
B[4]	> 20	5.6	11
C	0.12	0.074	11
D	0.079	0.042	8.6

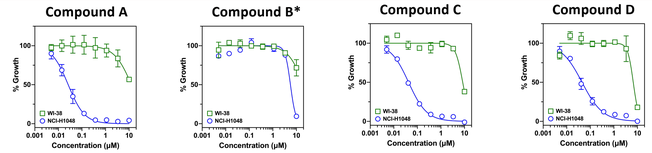
[1] Fluorescence polarization competitive assay
[2] 5-day proliferation MTT assay
[3] 3-day proliferation MTT assay
[4] Compound B is an inactive enantiomer of compound A

Figure 2. Cyclin A RxL inhibitors, exemplified by Compound D, exhibit no significant inhibition of kinase activity



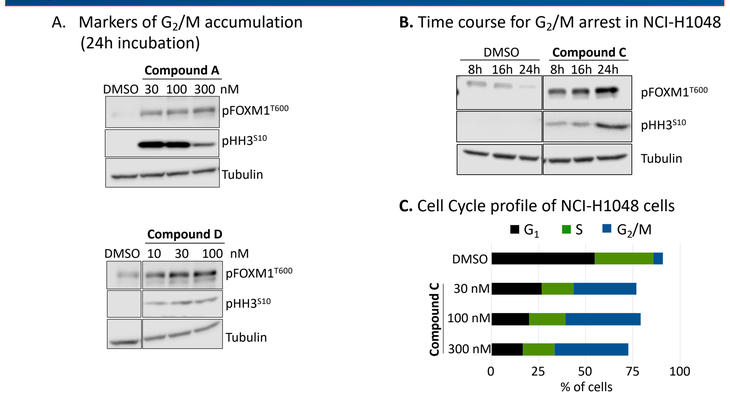
KINOME*scan*™ (Eurofins Discovery) screening for off-target activity with Compound D at 10 μM. Active site-directed competition binding assay quantifies interactions between test compound and a panel of 468 human kinases and kinases with tumor driver mutations. Shown is a representation of the human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

Figure 3. Cyclin A selective RxL inhibitors arrest NCI-H1048 SCLC cell line proliferation compared to WI-38, a normal human fibroblast cell line



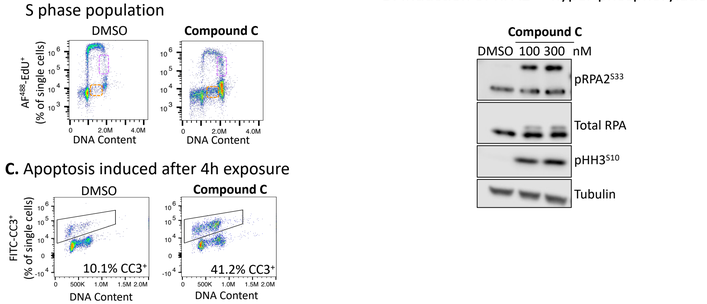
Dose response curves in NCI-H1048 and WI-38 normal fibroblast cell line. NCI-H1048 cells (blue lines) were plated in 96 well plates and exposed to compounds A — D for 5 days. WI-38 cells (green lines) were plated in 96 well plates and exposed to compounds A – D for 3 days. Cell growth was determined by MTT assay. See Table I for average growth inhibition 50 (GI_{50}) values. *Compound B is the inactive enantiomer of Compound A.

Figure 4. Cyclin A selective RxL inhibitors induce G_2/M phase arrest and accumulation of G_2/M phase markers, pHH3^{S10} and pFOXM1^{T600}, in NCI-H1048 SCLC cell line



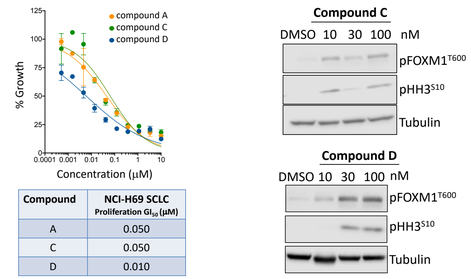
(A) Cyclin A RxL inhibitors induce markers of G_2/M accumulation. NCI-H1048 cells were incubated with DMSO, compound A, or compound D for 24h at the concentrations indicated. Cell lysates were analyzed by Western Blot. **(B) Up-regulation of G_2/M markers is observed with 8h of exposure to Cyclin A RxL inhibitors.** NCI-H1048 cells were incubated with DMSO or 300nM compound C for the times indicated. Cell lysates were analyzed by Western Blot. **(C) NCI-H1048 cell cycle profile.** NCI-H1048 cells were treated with compound C for 24h. 10μM EdU was added 1h before collection for flow cytometry. Cell cycle profile was assessed by detection of EdU (Click-it EdU) and DNA content (FxCycle dye).

Figure 5. Cyclin A selective RxL inhibitors induce apoptosis and affect markers of DNA damage in NCI-H1048 SCLC cells



(A) Evidence of DNA damage observed by EdU incorporation and cell cycle profiling. NCI-H1048 cells were incubated with DMSO or 300nM compound C for 24h. Cell cycle profile was assessed by EdU incorporation (10μM, 1h) combined with DNA content (FxCycle). Compound C induces an increase in the both the EdU negative-S phase population (orange box) and 4C DNA content (purple box). 4C DNA content is defined as cells with 4N DNA content undergoing active DNA replication (indicated by EdU positivity). **(B) Western blot analysis of phosphorylated RPA2, a biomarker for activation of the DNA damage response pathway.** NCI-H1048 cells were incubated for 24h with compound C at the concentrations indicated. Cell lysates were analyzed by Western blot. **(C) Cyclin A RxL inhibitors induce Caspase 3 activity.** NCI-H1048 cells were exposed to DMSO or 300nM compound C for 4h. Cells were washed and then incubated for an additional 40h before collecting for flow cytometry. Caspase 3 activity was assessed by detection of FITC-cleaved caspase 3 (CC3) and DNA content (FxCycle).

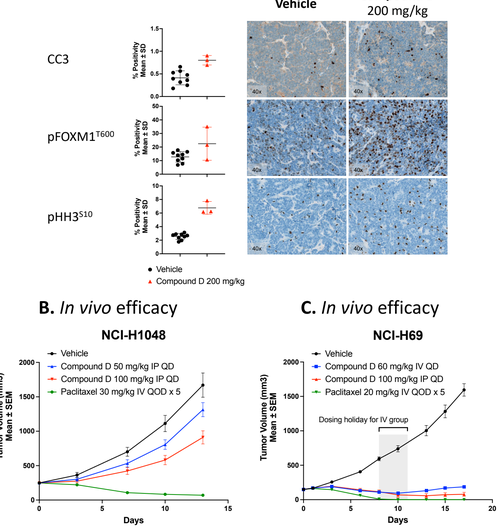
Figure 6. Cyclin A selective RxL inhibitors inhibit growth and induce markers of G_2/M phase arrest in NCI-H69 (ASCL1$^+$) SCLC cells



Compound	NCI-H69 SCLC Proliferation GI_{50} (μM)
A	0.050
C	0.050
D	0.010

(A) Dose response curves in NCI-H69 cells. NCI-H69 cells were plated in 96 well plates and exposed to compounds C and D for 5 days. Cell growth was determined by MTT assay. GI_{50} values for compounds A, C and D are reported in the table. **(B) Markers of G_2/M phase are up-regulated in NCI-H69 cells**. NCI-H69 cells were exposed to compound C or D at the indicated concentrations for 24h. Cell lysates were analyzed by Western blot.

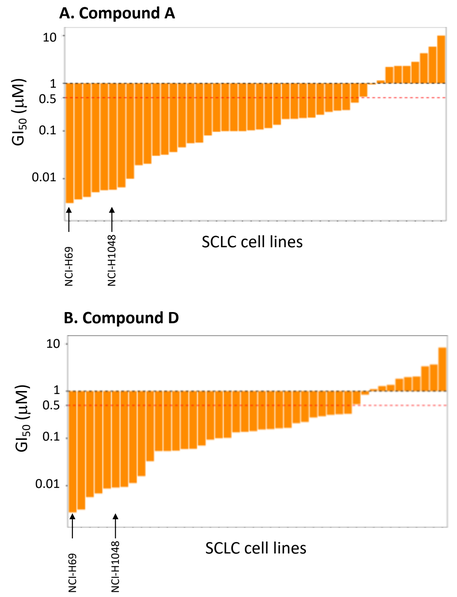
Figure 7. Cyclin A selective inhibition induces mitotic arrest and apoptosis and produces anti-tumor efficacy in *in vivo* SCLC models



(A), (B), and (C) *In vivo* pharmacodynamics and efficacy studies. (A) Mice were inoculated SC with 3x10^6 NCI-H1048 cells, then treated with a single dose of Vehicle or 200 mg/kg Compound D IP when tumors reached 200-300mm^3. Tumors were collected 16 hours post dose (n=6 for Vehicle, n=3 for Compound D), fixed, and stained for CC3, pFOXM1, and pHH3. **(B)** Mice were inoculated as in **Figure 7A**. IP dosing was initiated when tumors reached 200-300mm^3. Compound D was administered QD at 50 and 100 mg/kg (n=10 for all groups). **(C)** Mice were inoculated with 5x10^6 NCI-H69 cells, Drug treatment was initiated when tumors reached 88-200mm^3. Compound D was administered QD at 60 mg/kg IV and 100 mg/kg IP (n=10 for all groups). After 8 days, animals in the IV group were given a 3-day dosing holiday, then dosing was resumed QOD until study end.

SC, subcutaneous; IP, intraperitoneal; QD, once daily; SD, standard deviation; SEM, standard error of mean

Figure 8. Many SCLC cell lines exhibit sub-micromolar sensitivity to Cyclin A RxL inhibitors



(A) and (B) Waterfall plots of 44 SCLC cell lines show a substantial number that respond (as assessed by GI_{50}) to inhibition of Cyclin A RxL binding. (A) 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. **(B)** Cell lines were exposed to Compound D as described above for compound A. The order of cell lines in plots **(A)** and **(B)** is the same.

Conclusions

- Cyclin A RxL inhibitors bind to Cyclin A/CDK2 with nanomolar biochemical potency with no direct inhibition of protein kinase activity, including CDKs.
- Disruption of Cyclin A RxL binding in SCLC cell lines induces DNA damage and G_2/M arrest leading to apoptosis.
- Cyclin A RxL inhibitors exhibit sub-micromolar potency in a large panel of SCLC cell lines.
- Cyclin A RxL inhibitors show single agent tumor growth inhibition and tumor regression in SCLC tumor lines *in vivo*.
- Selective inhibition of cyclins offers a mechanistically distinct mode of action from strategies that inhibit cell cycle kinases, and is expected to be clinically useful.

References

1. Chen et al. (1999) *PNAS* 96, 4325–4329
2. Knudsen ES and Knudsen KE (2008) Nat. Rev. Cancer 8, 1-11
3. Mendoza et al. (2003) *Cancer Res.* 63, 1020–1024