1558. Targeting CDK9 via the small-molecule inhibitor enitociclib as a therapeutic strategy to treat *MYCN*-amplified rhabdomyosarcoma and neuroblastoma in children

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INTRODUCTION

- *MYCN* amplification is the genetic aberration most consistently associated with poor outcomes in alveolar rhabdomyosarcoma (aRMS) and neuroblastoma (NBL) in children, regulating key oncogenic processes including tumor cell survival, proliferation and metastasis.
- In aRMS, *MYCN* transcription is driven by the *PAX3-FOXO1* gene fusion, generated by the t(2;13) chromosomal translocation. *MYCN* is amplified and localized to super enhancer regions in NBL.
- CDK9, in complex with Cyclin T1, forms the positive transcription elongation factor, P-TEFb, that regulates RNA polymerase II (RNAPII) transcription initiation and elongation. RNAPII inhibition by CDK9 blockade leads to the disruption of transcription of *MYC* and other short half-life mRNA gene transcripts, providing durable control of oncogenic protein levels resulting in apoptosis and antitumor effects.
- The small-molecule inhibitor **enitociclib** (VIP152) targets P-TEFb/CDK9 and has shown effective molecular targetability and tolerability in preclinical and early phase clinical studies.[1-4]
- In this study, we demonstrate the efficacy of enitociclib as a single-agent *in vitro* in aRMS and NBL cell lines and in combination with several FDA-approved antineoplastic agents across a range of *in vitro aRMS* cell lines.

METHODS

- Cell-based viability assays were performed in several RMS (Rh30, Rh41 and RD) and NBL (LAN1, SK-N-AS, SK-N-BE(2) and SK-N-MC) cell lines.
  - The antitumor activity of enitociclib was determined by Alamar Blue cell viability assay after 96 h of treatment at 9 dose levels (8 nM–2 μM) with 1% DMSO (v/v) used as vehicle control.
  - A panel of targeted small-molecule inhibitors (n = 223) at a final concentration of 1 μM was used to screen and identify potentially active agents and pathways.
- Combination screens of enitociclib with several FDA-approved antineoplastic agents were analyzed.
  - Constant dilution ratios were used in pairwise or three-way drug combinations and drug synergy was calculated by the zero-interaction potency (ZIP) or Bliss models of synergy using SynergyFinder 3.0 software.
- Target modulation by enitociclib as a single-agent or in combination was determined for RNAPII, MYC, MCL1, PCNA, cleaved/pro-caspase-3 and PARP by western blotting after continuous exposure of the compounds for 24 h.
- Annexin V/propidium iodide (PI) staining was conducted to confirm apoptosis induction.
  - Rh30 and Rh41 cells were treated with 25-50 nM enitociclib or 1% DMSO (v/v) vehicle control for 24 h.
  - Late apoptotic cells were gated in the Q2 fraction.

REFERENCES

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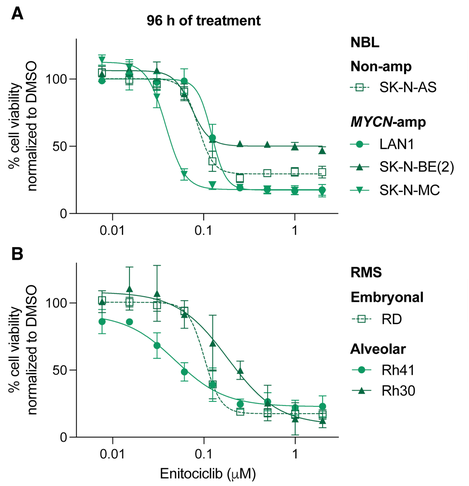
RESULTS

Enitociclib demonstrates cytotoxic activity *in vitro* against a panel of aRMS and NBL cell lines



Cell Line	*MYCN*-amp	Enitociclib IC_{50} (nM)
SK-N-AS	No	122.5
LAN1	Yes	87.0
SK-N-BE(2)	Yes	73.8
SK-N-MC	Yes	38.5

Cell Line	PAX3-FOXO1 Fusion	Enitociclib IC_{50} (nM)
RD	No	101.1
Rh41	Yes	48.3
Rh30	Yes	182.9

Figure 1. Dose-response curves of (A) neuroblastoma (NBL) or (B) rhabdomyosarcoma (RMS) cell lines treated with increasing concentrations (8 nM–2 μM) of enitociclib for 96 h. Cell viability was measured by Alamar Blue assay. Percent cell viability was normalized to corresponding treatment with DMSO (vehicle control). Mean percentages were calculated from three technical replicates and standard deviations are shown.

Enitociclib induces apoptosis and abates p-RNAPII Ser2 phosphorylation to provide therapeutic relief of key oncogenic markers in aRMS and NBL

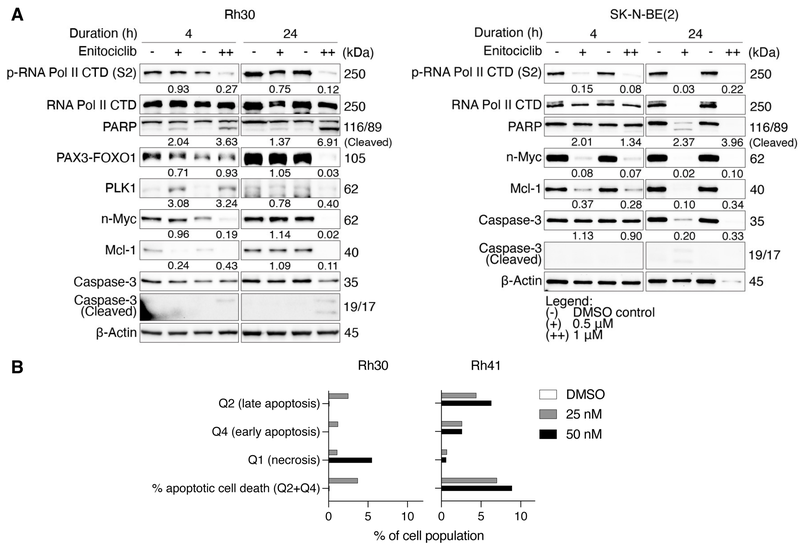


Figure 2. (A) Western blotting of Rh30 and SK-N-BE(2) cell line lysates treated with 0.5 μM/1 μM enitociclib (+/++) or DMSO vehicle control (-) for up to 24 h. Total cell lysates were probed for markers associated with RNAPII transcription (p-Ser2 and total RNAPII), short half-life mRNA gene transcripts (n-Myc and Mcl-1), apoptosis (PARP and caspase-3) and oncogenic markers of aRMS (PAX3-FOXO1 fusion and its stabilizing protein PLK1). β-actin was used as a loading control. Molecular masses are indicated in kilodaltons (kDa). **(B) Annexin V/PI staining of Rh30 and Rh41 aRMS cell lines following treatment with 25 nM–50 nM enitociclib or DMSO vehicle control for 24 h.**

Enitociclib demonstrates synergistic effects with several antineoplastic agents in preclinical models of aRMS

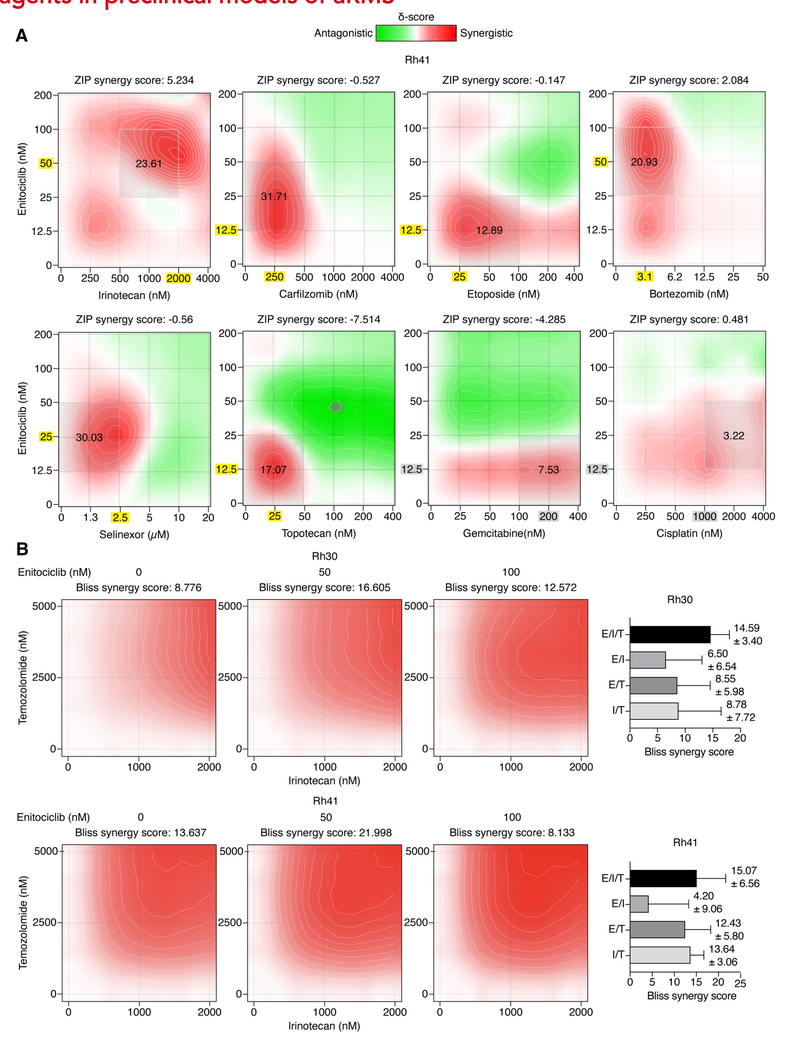


Figure 3. (A) 2-D response surface plots of pairwise combinatory activity for enitociclib with several FDA-approved antineoplastic agents against Rh41 aRMS cells. Cell viability was measured by Alamar Blue assay after 96 h of treatment. Percent cell viability was normalized to corresponding treatment with DMSO (vehicle control). Red indicates synergism; green indicates antagonism of the respective drug combinations. **Yellow = Maximum Synergistic Effect (MSE)** >10 ZIP synergy score (threshold for synergism). **(B) 2-D response surface plots of three-drug combinatory activity for enitociclib (50 nM–100 nM) with irinotecan (1 μM–2 μM) and temozolomide (2.5 μM–5 μM) in Rh30 and Rh41 cell lines for 96 h.** Measurements are averages of biological duplicates.

CONCLUSIONS

- Enitociclib is cytotoxic in aRMS and NBL cell lines at IC_{50} levels which are clinically achievable concentrations.
- Trends of sensitivity enrichment with *MYCN* amplification of PAX3-FOXO1 fusion is demonstrated.
- RNA Pol II phosphorylation as well as n-Myc and Mcl-1 proteins are downregulated in both aRMS and NBL cell lines resulting in apoptosis.
- Combinatory activity of enitociclib in aRMS with several FDA-approved treatments underpin that further investigation is warranted.

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