Preclinical Study of Enitociclib, a Selective CDK9 Inhibitor, in Combination with Bortezomib, Lenalidomide, Pomalidomide, or Venetoclax in the Treatment of Multiple Myeloma

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INTRODUCTION

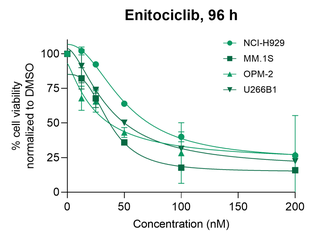
- Multiple Myeloma (MM) is a highly aggressive hematologic malignancy affecting plasma cells. Despite major advances in treatment, MM remains incurable, and a significant proportion of patients experience disease progression or relapse.
- Studies have shown that Cyclin D and *MYC* dysregulation together with downstream alterations in the apoptosis regulators are crucial in MM tumor cell survival, proliferation and treatment resistance.[1,2]
- 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/formerly BAY1251152) targets P-TEFb/CDK9 and has shown effective molecular targetability and tolerability in preclinical and early phase clinical studies in several tumor models.[3,4]
- Herein, we demonstrate the efficacy of enitociclib as a single-agent and in combination with anti-MM agents across a range of *in vitro* MM cell lines, as well as *in vivo* models of MM.

METHODS

- Cell-based viability assays were performed in several MM cell lines including NCI-H929, MM.1S, OPM-2 and U266B1.
  - The antitumor activity was determined by Alamar Blue cell viability assay after 96 h of treatment.
  - 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 anti-MM agents such as bortezomib, lenalidomide, pomalidomide and venetoclax were analyzed.
  - Constant dilution ratios of two inhibitors and drug synergy was calculated by the zero-interaction potency (ZIP) method 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.
- *In vivo* enitociclib experiments were conducted using MM cell lines JJN-3, NCI-H929 and OPM-2 in SCID/Beige mouse xenograft models.
  - Mechanism of action (MOA) was determined upon a single dose of 15 mg/kg enitociclib administered intravenously (IV).
  - Efficacy on tumor growth and survival was examined following a dosing schedule of 15 mg/kg enitociclib administered IV every 7 days and in combination with 50 mg/kg lenalidomide given orally daily.

RESULTS

Enitociclib identified as a top hit in small-molecule inhibitor screening and delivers cytotoxic activity *in vitro* against a panel of MM cell lines



- First, the small-molecule inhibitor screening identified enitociclib as a highly active agent in MM cells.
- Subsequently, exposure to enitociclib for 96 h against a representative panel of MM cell lines (n = 4) demonstrated significant cytotoxic activity, with IC_{50} values ranging from 36 nM to 78 nM (**Fig. 1**).

Figure 1. Dose-response curves of MM cell lines treated with increasing concentrations (25-200 nM) 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 of cell viability were calculated from three technical replicates and standard deviations are shown.

Cell Line	c-MYC Status	MCL1 Status	Enitociclib IC_{50} (nM)
NCI-H929	HOMDEL	AMP	78
MM.1S	-	-	36
OPM-2	-	AMP	41
U266B1	-	-	50

Target modulation of RNAPII and key markers associated with apoptosis after enitociclib treatment

- The induction of apoptosis was observed with cleavage of pro-caspase-3 and PARP by western blotting in a time-/dose-dependent manner with enitociclib as a single-agent, in addition to the depletion of p-RNAPII (Ser 2/5), MYC, MCL1 and PCNA proteins (**Fig. 2**).

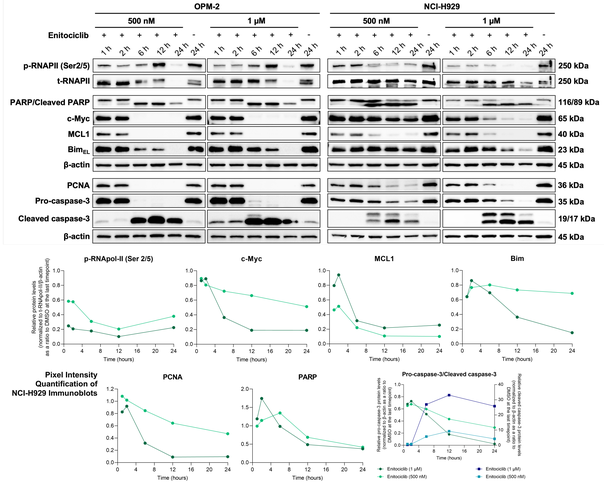


Figure 2. Western blotting of MM cell line lysates treated with either DMSO (vehicle control; "-") or 500 nM or 1 µM for 24 h. Total cell lysates were prepared and analyzed by immunoblotting to detect the levels of markers associated with apoptosis (total and cleaved PARP and caspase-3, MCL1 and Bim$_{EL}$), RNAPII transcription (phosphorylated serine 2/5 RNAPII and total RNAPII) and short-life mRNA gene transcripts (MYC and PCNA). β-actin was used as a loading control. Molecular masses are indicated in kilodaltons (kDa).

Enitociclib synergizes with several anti-MM agents at pharmacologically relevant concentrations

- SynergyFinder analysis detected synergistic effects of enitociclib in combination with bortezomib, lenalidomide, pomalidomide and venetoclax (synergy scores >10) at the tested concentrations (**Fig. 3**).

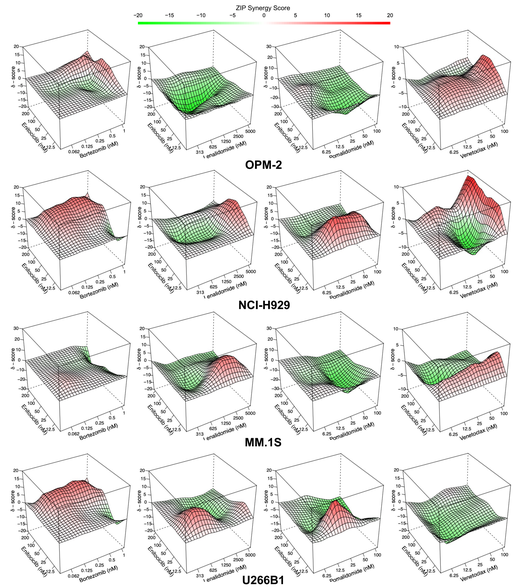


Figure 3. 3-D response surface plots of combinatory activity for enitociclib with bortezomib, lenalidomide, pomalidomide or venetoclax in MM cell lines. Cell viability was measured by Alamar Blue assay. Percent cell viability was normalized to corresponding treatment with DMSO (vehicle control).

Enitociclib enhances the efficacy of lenalidomide and venetoclax in preclinical models of MM

- Robust apoptosis induction through caspase-3 activation and PARP cleavage were observed with the presence of lenalidomide (**Fig. 4A**) or venetoclax (**Fig. 4B**) at 2 h in combination with enitociclib.

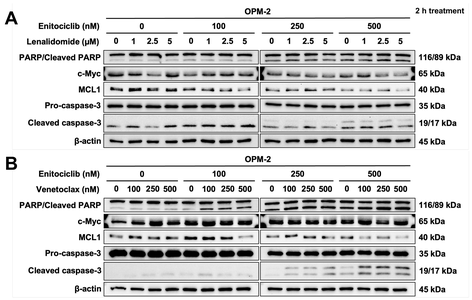


Figure 4. Western blotting of OPM-2 cell lysates treated with enitociclib in combination with lenalidomide or venetoclax for 2 h. Total cell lysates were prepared and analyzed by immunoblotting to detect the levels of markers associated with apoptosis (total and cleaved PARP and caspase-3 and MCL1) and short-life mRNA gene transcripts (MYC and PCNA). β-actin was used as a loading control. Molecular masses are indicated in kDa.

Enitociclib induces significant regression of MM xenograft tumors *in vivo*, downregulates *MYC* and induces apoptosis

- *In vivo*, IV administration of enitociclib transiently inhibits the transcription of *MYC* and *MCL1* and promotes apoptosis by induction of pro-caspase-3 and PARP cleavage with the onset of drug-induced effects seen as early as 1 h after enitociclib treatment (**Fig. 5**).

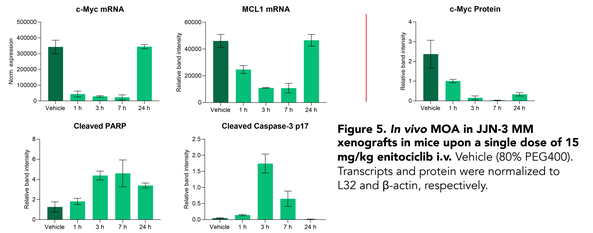


Figure 5. *In vivo* MOA in JJN-3 MM xenografts in mice upon a single dose of 15 mg/kg enitociclib i.v. Vehicle (80% PEG400). Transcripts and protein were normalized to L32 and β-actin, respectively.

- In enitociclib-treated mice, tumor volumes were reduced to 1-4% that of control mice on day 20 after the start of treatment (**Fig. 6A**), and increased efficacy of enitociclib in combination with lenalidomide was observed (**Fig. 6B**).
  - Enitociclib increased median survival time by up to 10.5 days.

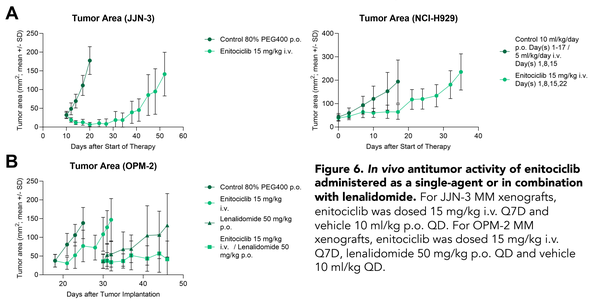


Figure 6. *In vivo* antitumor activity of enitociclib administered as a single-agent or in combination with lenalidomide. For JJN-3 MM xenografts, enitociclib was dosed 15 mg/kg i.v. Q7D and vehicle 10 ml/kg p.o. QD. For OPM-2 MM xenografts, enitociclib was dosed 15 mg/kg i.v. Q7D, lenalidomide 50 mg/kg p.o. QD and vehicle 10 ml/kg QD.

CONCLUSIONS

- Our studies present proof-of-concept evidence that enitociclib has significant antitumor activity against MM cells and provides specific pharmacological targetability of several key oncogenic pathways involving proteins such as MYC, MCL1 and PCNA, leading to growth inhibition and apoptosis.
- Taken together, the data provide the rationale and biological reasoning for further optimization studies of CDK9 inhibitors for clinical application and early phase clinical studies to improve outcomes in MM.

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Presented at the ASH Meeting 2022, December 10-13, 2022