blood neoplasia

American Society of Hematology
2021 L Street NW, Suite 900,
Washington, DC 20036
Phone: 202-776-0544 | Fax 202-776-0545
bloodneoplasia@hematology.org

Enitociclib, a selective CDK9 inhibitor: in vitro and in vivo preclinical studies in multiple myeloma

Tracking no: NEO-2024-000226R1

Son Tran(Oncology,University of Calgary,Canada;,University of Calgary,Canada) Patrick Sipila(Department of Oncology,University of Calgary,Canada) Melanie Frigault(,Vincerx Pharma,United States) Beatrix Stelte-Ludwig(,Vincerx Pharma,Germany) Amy Johnson(,Vincerx Pharma,United States) Joseph Birkett(,Vincerx Pharma,Germany) Raquel Izumi(,Vincerx Pharma,United States) Ahmed Hamdy(,Vincerx Pharma,United States) Ranjan Maity(,University of Calgary,;,University of Calgary,Canada) Nizar Bahlis(Department of Medicine, Hematology Oncology,Arnie Charbonneau Cancer Institute, University of Calgary,Canada;,University of Calgary,Canada) Paola Neri(Medicine,University of Calgary,Canada) Aru Narendran(,University of Calgary,Canada)

Abstract:
Multiple myeloma (MM) is a cancer of the plasma cells that remains incurable despite advances in treatment options. In this study, a library of 216 clinically feasible small-molecule inhibitors was screened to identify agents that selectively inhibit MM cell proliferation. Enitociclib, a CDK9-specific small-molecule inhibitor, was found to be highly effective at decreasing cell viability and inducing apoptosis in four MM cell lines. Enitociclib inhibited the phosphorylation of the CTD of RNA Pol II at Ser2/Ser5 and repressed the protein expression of oncogenes c-Myc, Mcl-1, and PCNA in MM cells. Additionally, enitociclib demonstrated synergistic effects with several anti-MM agents, including bortezomib, lenalidomide, pomalidomide, and venetoclax. These results suggest that enitociclib may represent a promising therapeutic option for the treatment of MM, either as a single agent or in combination with other anti-MM agents.

Conflict of interest: COI declared - see note

COI notes: MF, BSL, AJ, JB, RI, and AH report employment. All other authors declare no competing financial interests.

Preprint server: No;

Author contributions and disclosures: All authors contributed to the conceptualization and design of the study. AN, PN, and NB provided supervision. ST, PS, RM, BSL, and JB performed experiments and analyzed data. ST, MF, BSL, and AJ wrote and edited the manuscript and PS revised the final version. All authors reviewed and approved the final manuscript.

Non-author contributions and disclosures: No;

Agreement to Share Publication-Related Data and Data Sharing Statement: Data may be requested from the corresponding author, Aru Narendran (a.narendran@ucalgary.ca).

Clinical trial registration information (if any):

Enitociclib, a selective CDK9 inhibitor: in vitro and in vivo preclinical studies in multiple myeloma

Running title: CDK9 inhibitor enitociclib in multiple myeloma

Son Tran[1], Patrick Sipila[1], Melanie M. Frigault[2], Beatrix Stelte-Ludwig[3], Amy J. Johnson[2], Joseph Birkett[3], Raquel Izumi[2], Ahmed Hamdy[2], Ranjan Maity[4], Nizar J. Bahlis[4], Paola Neri[4], Aru Narendran[1]

[1] Department of Oncology, University of Calgary, Calgary, AB, Canada

[2] Vincerx Pharma, Inc., Palo Alto, CA, USA

[3] Vincerx Pharma GmbH, Monheim, Germany

[4] Departments of Hematology and Oncology, University of Calgary, Calgary, AB, Canada

Corresponding author: Aru Narendran; University of Calgary, 3330 Hospital Dr NW, HMRB 326; Calgary, AB, Canada, T2N 4N1; e-mail: a.narendran@ucalgary.ca; phone: (403) 210-6418

Text word count: 2,938

Abstract word count: 133

Figure/table count: 5 figures, 3 supplementary figures, 2 supplementary tables

Reference count: 24

CDK9 inhibitor enitociclib in multiple myeloma

Key Points

- Targeting CDK9 with enitociclib induces apoptotic cell death in multiple myeloma and demonstrates synergy in drug combinations
- Enitociclib may be an effective treatment for multiple myeloma as a single agent or in combination therapies in future clinical studies

Abstract

Multiple myeloma (MM) is a cancer of the plasma cells that remains incurable despite advances in treatment options. In this study, a library of 216 clinically feasible small-molecule inhibitors was screened to identify agents that selectively inhibit MM cell proliferation. Enitociclib, a CDK9-specific small-molecule inhibitor, was found to be highly effective at decreasing cell viability and inducing apoptosis in four MM cell lines. Enitociclib inhibited the phosphorylation of the CTD of RNA Pol II at Ser2/Ser5 and repressed the protein expression of oncogenes c-Myc, Mcl-1, and PCNA in MM cells. Additionally, enitociclib demonstrated synergistic effects with several anti-MM agents, including bortezomib, lenalidomide, pomalidomide, and venetoclax. These results suggest that enitociclib may represent a promising therapeutic option for the treatment of MM, either as a single agent or in combination with other anti-MM agents.

Introduction

Multiple myeloma (MM) is a blood cancer characterized by the uncontrolled growth of clonal plasma cells.[1] This leads to clinical symptoms such as hypercalcemia, renal failure, anemia, and bone lesions. The disease follows a multistep process, starting with premalignant monoclonal gammopathy of undetermined significance, progressing through smoldering multiple myeloma, and then reaching active MM. The progression of MM is associated with the accumulation of genomic alterations and immune dysfunction, which increases the risk of infectious complications and promotes tumor progression. Anticancer efficacy has greatly improved in recent years, with 5-year overall survival now exceeding 50%.[2] Despite significant progress in MM treatment, active disease remains incurable. Aberrant expression of cyclin-dependent kinases (CDKs) plays a role in MM by causing loss of control over cell proliferation and enhancing survival. CDK9, a serine/threonine kinase and a subunit of the positive transcription elongation factor (P-TEFb), is a major transcriptional regulator of various oncogenes relevant in the pathogenesis of MM, including *MYC*, *MCL1*, and *PCNA*.[3,4] Understanding the role of CDKs in MM could potentially lead to more effective treatments for this disease.

The small molecule enitociclib (VIP152) is a CDK9 inhibitor currently undergoing early phase clinical trials for various hematological malignancies, such as diffuse large B cell lymphoma.[5,6] We hypothesized that CDK9 inhibition by enitociclib would represent a rational pharmacologic approach to target the transcription of critical oncogenic effector proteins in MM. In this study, we describe that enitociclib induces cytotoxicity *in vitro* and responses *in vivo* against aggressive MM cells. The responses were due to the downregulation of RNA polymerase II-mediated transcription of MM-related oncogenes, including *MYC*, *MCL1*, and *PCNA*,

followed by rapid drug-induced apoptosis. The potential clinical benefits of enitociclib warrant further investigation in the context of treating patients with MM.

Methods

Cell culture

Multiple myeloma cell lines NCI-H929 [DSMZ Cat# ACC-163, RRID: CVCL_1600], MM1.S [ATCC Cat# CRL-2974; RRID: CVCL_8792], OPM-2 [DSMZ Cat# ACC-50; RRID: CVCL_1625], and U266B1 [ATCC Cat# TIB-196; RRID: CVCL_0566] were cultured in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin/L-glutamine (Gibco) at 37°C and 5% CO_2. JJN-3 myeloma cells [DSMZ Cat# ACC-541, RRID: CVCL_2078] were cultured in 50% DMEM (Biochrom)/50% IMDM (Gibco) supplemented with 10% FBS (Gibco).

Cytotoxicity assays

Cells were seeded in 96-well plates (Greiner Bio-One, Monroe, NC, USA) at 5×10^3 cells per well in 100 µl of RPMI-1640 media (Gibco) supplemented with 10% FBS. The cells were treated with either enitociclib or DMSO diluted in media and 100 µl was added to each well (1:2 final dilution). All treatments were run in triplicate at final concentrations ranging from 12.5–200 nM. After 96 hours in culture, cell viability was evaluated using the alamarBlue® Cell Viability (Alamar Blue) reagent (Thermo Fisher Scientific) as per manufacturer's instructions. Half-maximal inhibitory concentrations (IC_{50}) were determined by non-linear regression using GraphPad Prism v9.1.0 analysis software (GraphPad Software, Inc., San Diego, CA, USA). Matrix dosing of agents in combinations was used to assess drug synergy utilizing the SynergyFinder v3.0 online web application tool (https://synergyfinder.fimm.fi/).[7] Synergy scores were determined by the zero-interaction potency (ZIP) method.

Small-molecule inhibitor screening

Cells were seeded in 96-well plates (Greiner Bio-One) at 5×10^3 cells per well and treated with a library containing 216 small-molecule compounds or corresponding DMSO treatment at a final concentration of 1 μM. After 96 hours, cell viability was measured using Alamar Blue reagent.

Western blotting

MM cells were lysed in RIPA buffer supplemented with 0.1% protease/phosphatase inhibitors. Protein concentration was quantified using the DC Protein Assay (Bio-Rad, Hercules, California, USA) as per manufacturer's instructions. Appropriate volumes of samples containing 30 μg of protein were mixed with loading buffer (0.125 M Tris-HCL [6.8 pH], 20% glycerol, 10% 2-mercaptoethanol, 4% SDS, 0.005% bromophenol blue). Samples were then resolved by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) using various percentages of acrylamide (7.5%-12%), depending on the protein of interest. SDS-PAGE was performed in running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS), followed by transfer to nitrocellulose membranes in 1X Trans-Blot Turbo transfer buffer (Bio-Rad) at 25 V for 10 minutes at room temperature using the Trans-Blot Turbo transfer system (Bio-Rad). The membranes were blocked in 5% (w/v) skim milk or bovine serum albumin serum (BSA) in Tris-buffered saline with 0.1% (v/v) Tween-20 (TSB-T; 50mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% [v/v] Tween-20) at room temperature for 1 hour. The membranes were then incubated overnight at 4°C with the following primary antibodies diluted in TBS-T with 5% (w/v) skim milk or BSA: anti-caspase-3 (1:1000, 9662S), anti-poly-(ADP-ribose) polymerase (PARP; 1:1000, 9542S), anti-Bim (1:1000, 2933S), anti-c-Myc (1:1000, 18583S), anti-MCL1 (1:1000, 94296S), anti-PCNA (1:1000, 13110S), anti-phospho-Rpb1 CTD (Ser2/Ser5; 1:1000, 13546S), anti-Rpb1 CTD

(1:1000, 2629S), and anti-β-actin (1:1000, 4967S) (all from Cell Signalling Technology, Whitby, ON, Canada). After, the membranes were washed three times with TBS-T, incubated with anti-rabbit secondary antibody (1:10000, 7074S, Cell Signalling Technology), washed three times with TBS-T, incubated with Clarity Western ECL substrates (Bio-Rad), and developed using chemiluminescence on a ChemiDoc MP imaging system (Bio-Rad).

Gene expression quantification

Total RNA was extracted from homogenized frozen tumor with RNeasy Plus Mini kit (QIAGEN, Germany) from two animals at 1, 3, 7, and 24 hours post-*in vivo* dosing and from three pre-dose animals. The quality and concentration of the RNA samples was tested by ultraviolet absorption at 260/280 nm in Nano Drop system and electrophoresis on denaturized 1% agarose gel to determine the concentration. *BCL2*, *CCND1*, *MYC*, *MCL1*, *HEXIM1*, and *PCNA* mRNA were quantified using TaqMan probes (Thermo Fisher Scientific) as per manufacturer's instructions. The real-time PCR results are expressed as the mean from three independent experiments for vehicle-treated animals and two independent experiments for drug-treated animals. Readouts were normalized to L32 transcript primers.

Animal studies

All animal studies were conducted at Bayer Pharma AG in accordance with German animal welfare law and approval by local authorities. For JJN-3 xenografts, 5.0×10^6 cells in 0.1 ml 50% Matrigel suspension were injected subcutaneously into SCID/Beige mice (Taconic Biosciences) and treated with 10 ml/kg 80% PEG400 control or 15 mg/kg enitociclib intravenously once per week. For OPM-2 xenografts, 5.0×10^6 cells in 0.1 ml medium suspension were injected subcutaneously into NOD/SCID mice (Taconic Biosciences) and treated with 5 ml/kg PEG400-ethanol-water (60/10/30) vehicle control or 15 mg/kg enitociclib

once per week by intravenous injection. For NCI-H929 xenografts, 3-4 mm tumor fragments obtained from serially passaged xenografts were implanted subcutaneously in the flank of NOD/SCID mice (Harlan Laboratories, The Netherlands) and treated with 5 ml/kg PEG400-ethanol-water (30/10/60) vehicle control or 15 mg/kg enitociclib once per week by intravenous injection. For drug combinations, mice bearing OPM-2 xenografts were treated with 50 mg/kg lenalidomide (vehicle: 10 ml/kg 1% cMC/0.5% Tween in water) daily by oral gavage, 0.8 mg/kg bortezomib (vehicle: 10 ml/kg saline) twice per week by intraperitoneal injection, or lenalidomide/bortezomib in combination with 15 mg/kg enitociclib as described above. Animals were monitored daily and two-dimensional tumor measurements were determined twice weekly using calipers.

Statistical analysis

The student's t-test or analysis of variance (ANOVA) statistical analyses were performed using GraphPad Prism v9.1.0 software. *P* values < 0.05 were deemed statistically significant.

Results

Enitociclib inhibits multiple myeloma cell proliferation and induces apoptosis *in vitro*

We examined the activity of agents from a library comprised of 216 individual drugs including clinically feasible small molecule inhibitors against various components of cancer growth and survival pathways. In this experiment, OPM-2 cells were treated with 1 μM of each therapeutic agent, followed by cell viability measurement under each experimental condition by Alamar Blue assay after 96 hours in culture (Supplementary Table 1). OPM-2 cells show high sensitivity to CDK inhibitors (Figure 1A), with CDK9-specific inhibition from enitociclib identified as a candidate agent due to its inhibition of oncogenes expressed in MM (Figure 1B).

To further investigate the anti-myeloma activity of enitociclib, we tested various concentrations in four MM cell lines that express CDK9 (Figure 1C) and different molecular features (Supplementary Table 2) with a range of enitociclib concentrations from 12.5–200 nM for 96 hours and cell viability was measured using the Alamar Blue assay. Enitociclib decreased cell viability in a concentration-dependent manner in all cell lines tested, with IC_{50} values ranging from 36–78 nM (Figure 1D). Notably, enitociclib exhibited similar activity in cells with *MCL1* gene amplification and cells without chromosome 1q gains. We also found that enitociclib potently induced mechanism-based apoptosis in NCI-H929 and OPM-2 cells, which have distinct molecular alterations. Western blot analysis showed that cleavage of apoptosis markers caspase-3 and PARP proteins occurred in a dose- and time-dependent manner, with significant activity observed as early as 6 hours after treatment at the tested concentrations (Figure 2). Likewise, persistent repression of the anti-apoptosis marker Mcl-1 was observed from 6 hours onwards post-treatment at both dose levels (Figure 2).

Enitociclib treatment leads to inhibition of CTD phosphorylation and short half-life transcript proteins

To confirm that the anti-proliferative effects of enitociclib were due to on-target inhibition of CDK9, we next examined the effects of CDK9-mediated transcriptional regulation in NCI-H929 and OPM-2 cells. The selectivity of enitociclib to inhibit the phosphorylation of the CTD of RNA Pol II at Ser2 (p-RNA Pol II S2) has been shown previously,[6,8] and the results showed that enitociclib could potently block the CTD phosphorylation of p-RNA Pol II S2/S5 in a dose- and time-dependent manner (Figure 2). We next investigated the impact of enitociclib treatment on the expression of oncogenes that are known to drive the progression of MM. Regarding the MM cell lines used in this study, *MYC* is highly expressed in NCI-H929 and

OPM-2 cells.[3] We found that treatment with enitociclib repressed c-Myc protein levels in NCI-H929 and OPM-2 cells (Figure 2). Enitociclib also repressed protein expression of proliferating cell nuclear antigen (PCNA) in these cells, which is a known disease-related factor in MM that is associated with cellular proliferation and cancer progression.[9] Together, these findings suggest that enitociclib exerts its anti-proliferative effects in MM cells by inhibiting CDK9-mediated transcriptional regulation of oncogenes.

CDK9-selective inhibition by enitociclib synergizes with several anti-multiple myeloma agents *in vitro*

Provided the ability of enitociclib to disrupt higher-order transcriptional machinery through selective CDK9 inhibition,[8] we hypothesized that the downregulation of molecular factors related to MM-related and antiapoptotic-driven drug resistance may enable effective combinatory activity with other chemotherapeutic agents. Thus, we evaluated the potential of enitociclib to synergize with current conventional and experimental agents used in the treatment of MM. A panel of MM cell lines were treated with increasing concentrations of enitociclib and one of four different agents, including bortezomib, lenalidomide, pomalidomide, and venetoclax, for 96 hours in culture before cell viability measurement using the Alamar Blue assay (Supplementary Figure 1).

The proteasome inhibitor bortezomib is U.S. Food and Drug Administration (FDA)-approved for first-line therapy in MM.[10] Despite high initial response rates, bortezomib eventually loses its efficacy. Therefore, we next determined if enitociclib could enhance the activity of bortezomib in MM cells. In OPM-2 cells, synergistic effects (>10 ZIP synergy score) were observed with 50 nM of enitociclib in combination with bortezomib treated at 1 nM (Figure 3). Lenalidomide is another FDA-approved first-line therapy in newly diagnosed MM, which

acts as both an immunomodulatory and anticancer drug by altering proinflammatory cytokine production and exerting antiproliferative and proapoptotic effects on MM cells through transcriptional control of MYC and other oncogenes.[11,12] When used in combination with enitociclib, synergistic effects were identified in three out of four cell lines tested (MM1.S, NCI-H929, and U266B1) at enitociclib concentrations between 12.5–25 nM and lenalidomide concentrations between 313 nM–5 μM (Figure 3). Pomalidomide is an FDA-approved immunomodulatory drug for patients with refractory MM, who have been treated with two prior therapies including lenalidomide and bortezomib. The drug acts by inhibiting cell proliferation and promoting apoptosis in MM cells, in addition to antiangiogenic properties.[13] NCI-H929 and U266B1 cells showed synergistic effects of enitociclib with pomalidomide at 12.5 nM and 12.5–50 nM for each agent, respectively (Figure 3). Lastly, the selective B-cell lymphoma 2 (Bcl-2) inhibitor venetoclax is an experimental drug for MM that has demonstrated antiproliferative activity in patients with relapse/refractory MM positive for t(11;14) translocation.[14] In general, enitociclib with venetoclax showed modest additive effects (ZIP synergy scores less than 10) across all four cell lines (Figure 3).

We then sought to examine the changes in protein expression levels of MM-related oncogenes and apoptosis markers in response to the two-drug combinations in a representative MM cell. Interestingly, we observed a dose-dependent repression of c-Myc protein in OPM-2 cells treated in combination with enitociclib and pomalidomide or venetoclax (Figure 4). The most robust inhibition of Mcl-1 protein was observed for the venetoclax combination in a dose-dependant manner in OPM-2 cells, which has *MCL1* gene amplification (Figure 4). Increased cleavage of PARP and caspase-3 were also seen in these combinations in a dose-dependent

manner (Figure 4). Overall, these data suggest clinical potential for enitociclib to be used in combination with conventional anti-MM agents.

Enitociclib shows mechanism-based efficacy *in vivo* as a single-agent and in combination against murine models of MM

To determine the mechanism of action of enitociclib *in vivo*, a single dose of 15 mg/kg enitociclib was administered intravenously in mice bearing JJN-3 cells and tumors were extracted at several timepoints up to 24 hours. Enitociclib was able to transiently inhibit the transcription of *MYC*, *MCL1*, and *PCNA* (Figure 5A) and promote apoptosis by the induction of caspase-3 and PARP cleavage (Figure 5B) with the onset of drug-induced effects observed as early as one hour after treatment. Protein levels of c-Myc were also abated for up to 24 hours (Figure 5B). Interestingly, *BCL2* levels remained consistent following treatment (Supplementary Figure 2). These results suggest that enitociclib maintains its mechanistic efficacy in MM when administered intravenously.

Next, we sought to assess the efficacy of enitociclib to reduce tumor burden *in vivo* to better evaluate its potential clinical utility in patients with MM. Murine models of MM were generated using JJN-3, NCI-H929, and OPM-2 cells that readily established xenograft tumors. Mice were randomized to vehicle versus enitociclib at the time disease was evident and treated until endpoint. Enitociclib was dosed 15 mg/kg administered intravenously once per week. As a single agent, enitociclib-treated mice had reduced tumor volumes and prolonged survival time compared to vehicle-treated mice (Figure 5C). To examine the therapeutic benefit of combining enitociclib with conventional anti-MM agents, mice bearing OPM-2 cells were randomly administered with lenalidomide (50 mg/kg taken orally daily), bortezomib (0.8 mg/kg intraperitoneally twice weekly), or the respective combination with enitociclib. Increased

efficacy of enitociclib in combination with lenalidomide or bortezomib was observed (Figure 5D). No significant changes in weight were observed with treatment (Supplementary Figure 3). These data provide evidence that the anti-MM activity of enitociclib is maintained *in vivo* and provides effective therapeutic relief by delaying tumor cell growth.

Discussion

We took a candidate approach to determining the anti-MM mechanism of action of the CDK9-specific small-molecule inhibitor enitociclib. Our small-molecule library screening approach revealed that MM cells were remarkably sensitive to single-agent CDK inhibitors, particularly CDK9 inhibition, providing rationale for this study to characterize and investigate the molecular mechanism of activity of enitociclib in MM. Activity by enitociclib in MM cells was similar to the IC_{50} values reported in lymphoma and leukemia cells.[6,8] We found that enitociclib potently and directly inhibited CDK9-mediated phosphorylation of RNA polymerase II S2/S5 and repressed the expression of oncogenes that are crucial for MM cell survival and proliferation. Combinatory approaches can reduce the amount of drug required to produce an anticancer effect, therefore lessening the potential of on-target and off-target drug toxicities. We explored a panel of conventional anti-MM drugs in combination with enitociclib and found that varying additive or synergistic interactions were observed in each combination at clinically relevant concentrations. However, antagonistic effects at certain concentrations were also noted in some cell lines, highlighting the importance of optimizing the dose range for each combination. Notably, enitociclib reduced the expression of *MCL1*, which has been shown to confer sensitivity to venetoclax.[15,16] Accordingly, CDK9 inhibition has also demonstrated synergy with venetoclax in leukemia models.[17] Lastly, enitociclib showed significant *in vivo*

efficacy as a single agent and in combination with lenalidomide or bortezomib across multiple murine MM xenograft models, and its mechanism of action was recapitulated *in vivo*.

MM cells are highly sensitive to enitociclib. The high sensitivity may be due to the instability of oncoproteins expressed in MM cells, which require perpetual replenishment through *de novo* synthesis.[18,19] As a result, their expression and activity may be significantly affected by an agent that disrupts transcription. Enitociclib inhibits CDK9 function, leading to a disruption in gene transcription.[20] This disruption results in a quick degradation of MM oncoproteins because of their instability and short protein half-lives. Since MM cells are highly dependent on these oncogenic signals, their loss of expression leads to rapid apoptosis. Therefore, enitociclib shows promise as a potential treatment option for MM due to its ability to disrupt transcription and rapidly degrade MM oncoproteins, resulting in the induction of apoptosis.

The results of our studies are the first to demonstrate that enitociclib has anti-MM activity. It also targets specific oncogenic pathways related to oncoproteins such as c-Myc, Mcl-1, and PCNA, resulting in the suppression of growth and the rapid induction of apoptosis. We also identify potential effective combinations for use with enitociclib in MM. These results demonstrate that enitociclib is effective in cell lines with unfavorable prognostic factors, such as p53 deletion, t(4; 14) and not restricted to the chromosome 1q gain subset where one of the genes at the 1q21 locus is *MCL1*.[21-23] These findings provide a solid basis and biological rationale for conducting further optimization studies of CDK9 inhibitors, which could have important clinical implications for improving outcomes in patients with MM.

Acknowledgements

This study was supported by funding from the KCC foundation and in part by a research grant from Vincerx Pharma to the study laboratory that was administered by the University of Calgary. Visual abstract created with BioRender.com.

Authorship

Contributions: All authors contributed to the conceptualization and design of the study. AN, PN, and NB provided supervision. ST, PS, RM, BSL, and JB performed experiments and analyzed data. ST, MF, BSL, and AJ wrote and edited the manuscript and PS revised the final version. All authors reviewed and approved the final manuscript.

Conflict of Interest Disclosures: MF, BSL, AJ, JB, RI, and AH report employment. All other authors declare no competing financial interests.

Correspondence: Aru Narendran; University of Calgary, 3330 Hospital Dr NW, HMRB 326; Calgary, AB, Canada, T2N 4N1; e-mail: a.narendran@ucalgary.ca; phone: (403) 210-6418

Data Sharing Statement: Data may be requested from the corresponding author, Aru Narendran (a.narendran@ucalgary.ca).

References

1. Palumbo A, Anderson K. Multiple myeloma. *N Engl J Med*. 2011;364(11):1046-60.
2. Turesson I, Bjorkholm M, Blimark CH, Kristinsson S, Velez R, Landgren O. Rapidly changing myeloma epidemiology in the general population: Increased incidence, older patients, and longer survival. *Eur J Haematol*. 2018;101(2):237–244.
3. Shou Y, Martelli ML, Gabrea A, et al. Diverse karyotypic abnormalities of the c-myc locus associated with c-myc dysregulation and tumor progression in multiple myeloma. *Proc Natl Acad Sci USA*. 2000;97(1):228–233.
4. Widden H, Placzek WJ. The multiple mechanism of MCL1 in the regulation of cell fate. *Commun Biol*. 2021;4:1029.
5. Diamond JR, Boni V, Lim E, et al. First-in-human dose-escalation study of cyclin-dependent kinase 9 inhibitor VIP152 in patients with advanced malignancies shows early signs of clinical efficacy. *Clin Cancer Res*. 2022;28(7):1285–1293.
6. Frigault MM, Mithal A, Wong H, et al. Enitociclib, a selective CDK9 inhibitor, induces complete regression of MYC+ lymphoma by downregulation of RNA polymerase II mediated transcription. *Cancer Res Commun*. 2023;3(11):2268-2279.
7. Ianevski A, Giri AK, Aittokallio T. SynergyFinder 3.0: an interactive analysis and consensus interpretation of multi-drug synergies across multiple samples. *Nucleic Acids Res*. 2022;50(W1):W739-W743.
8. Sher S, Whipp E, Walker J, et al. VIP152 is a selective CDK9 inhibitor with pre-clinical in vitro and in vivo efficacy in chronic lymphocytic leukemia. *Leukemia*. 2023;37(2):326–338.

9. Tsirakis G, Pappa CA, Kaparou M, et al. Assessment of proliferating cell nuclear antigen and its relationship with proinflammatory cytokines and parameters of disease activity in multiple myeloma patients. *Eur J Histochem*. 2011;55(3):e21.
10. Field-Smith A, Morgan GJ, Davies FE. Bortezomib (Velcade™) in the treatment of multiple myeloma. *Ther Clin Risk Manag*. 2006;2(3):271–279.
11. Bird S, Pawlyn C. IMiD resistance in multiple myeloma: current understanding of the underpinning biology and clinical impact. *Blood*. 2023;142(2):131-140.
12. Neri P, Barwick BG, Jung D, et al. ETV4-dependent transcriptional plasticity maintains MYC expression and results in IMiD resistance in multiple myeloma. *Blood Cancer Discov*. 2024;5(1):56-73.
13. Chanan-Khan AA, Swaika A, Paulus A, et al. Pomalidomide: the new immunomodulatory agent for the treatment of multiple myeloma. *Blood Cancer J*. 2013;3(9):e143.
14. Kumar S, Kaufman JL, Gasparetto C, et al. Efficacy of venetoclax as targeted therapy for relapsed/refractory t(11;14) multiple myeloma. *Blood*. 2017;130(22):2401–2409.
15. Ten Hacken E, Valentin R, Regis FFD, et al. Splicing modulation sensitizes chronic lymphocytic leukemia cells to venetoclax by remodeling mitochondrial apoptotic dependencies. *JCI Insight*. 2018;3(19):e121438.
16. Guièze R, Liu VM, Rosebrock D, et al. Mitochondrial reprogramming underlies resistance to BCL-2 inhibition in lymphoid malignancies. *Cancer Cell*. 2019;36(4):369-384.e13.

17. Chen R, Chen Y, Xiong P, et al. Cyclin-dependent kinase inhibitor fadraciclib (CYC065) depletes anti-apoptotic protein and synergizes with venetoclax in primary chronic lymphocytic leukemia cells. *Leukemia*. 2022;36(6):1596-1608.
18. Steward DP, Koss B, Bathina M, Perciavalle RM, Bisanz K, Opferman JT. Ubiquitin-independent degradation of antiapoptotic MCL-1. *Mol Cell Biol*. 2010;30(12):3099–3110.
19. Gregory MA, Hann SR. c-Myc proteolysis by the ubiquitin-proteasome pathway: stabilization of c-Myc in Burkitt's lymphoma cells. *Mol Cell Biol*. 2000;20(7):2423–2435.
20. Lucking U, Kosemund D, Bohnke N, et al. Changing for the better: discovery of the highly potent and selective CDK9 inhibitor VIP152 suitable for once weekly intravenous dosing for the treatment of cancer. *J Med Chem*. 2021;64(15):11651–11674.
21. Schmidt TM, Fonseca R, Usmani SZ. Chromosome 1q21 abnormalities in multiple myeloma. *Blood Cancer J*. 2021;11:83.
22. Sato S, Kamata W, Okada S, Tamai Y. Clinical and prognostic significance of t(4;14) translocation in multiple myeloma in the era of novel agents. *Int J Hematol*. 2021;113:207–213.
23. Martello M, Poletti A, Borsi E, et al. Clonal and subclonal TP53 molecular impairment is associated with prognosis and progression in multiple myeloma. *Blood Cancer J*. 2022;12:15.
24. Wallington-Beddoe CT, Mynott RL. Prognostic and predictive biomarker developments in multiple myeloma. *J Hematol Oncol*. 2021;14:151.

Figure Legends

Figure 1. Enitociclib decreases cell viability in MM cells. (A) Cell viability of small-molecule inhibitors from the pharmaceutical pipeline library treated at 1 μM for 96 hours in OPM-2 cells. A breakdown of molecular targets with a mean cell viability inhibition of >50% are shown. (B) Cell viability values of various CDK inhibitors from the library treated at 1 μM for 96 hours in OPM-2 cells are shown. (C) Western blotting of MM cell lines MM1.S, NCI-H929, OPM-2, and U266B1. Total cell lysates were prepared and analyzed by immunoblotting to detect the level of CDK9. β-actin was used as a loading control. Molecular masses are indicated in kilodaltons (kDa). (D) Dose response curves of MM cell lines treated with increasing concentrations (12.5–200 nM) of enitociclib for 96 hours. 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 form three technical replicates and standard deviations are shown.

Figure 2. Enitociclib induces apoptosis by inhibition of RNA Pol II phosphorylation and oncogene expression in MM cells. Western blotting of NCI-H929 and OPM-2 MM cells treated with either DMSO (vehicle control; '-') or 0.5–1 μM of enitociclib for up to 24 hours. Total cell lysates were prepared and analyzed by immunoblotting to detect levels of markers associated with apoptosis (total and cleaved PARP and caspase-3, Mcl-1, and Bim_{EL}), total and phosphorylated RNA Pol II CTD (S2/S5), and short half-life oncogene proteins c-Myc and PCNA. β-actin was used as a loading control. Molecular masses are indicated in kDa.

Figure 3. Enitociclib is synergistic with anti-MM chemotherapies. 3D response surface plots for combinatory activity of enitociclib with bortezomib, lenalidomide, pomalidomide, or venetoclax treated in MM cell lines for 96 hours. Cell viability was measured by Alamar Blue

assay. Percent cell viability was normalized to corresponding treatment with DMSO (vehicle control). Synergy score is calculated by SynergyFinder based on the ZIP interaction model.[7] Red indicates synergism and green indicates antagonism of the respective drug combinations. Maximal synergistic effects (MSE) are indicated where the synergy score is greater than 10 and the drug concentrations at which the MSE occurs are highlighted in yellow.

Figure 4. Enitociclib enhances apoptosis and oncogene repression. Western blotting of OPM-2 cells treated with enitociclib in combination with bortezomib, lenalidomide, pomalidomide, or venetoclax for 6 hours. 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 Mcl-1) and short half-life oncogene proteins (c-Myc and PCNA). β-actin was used as a loading control. Molecular masses are indicated in kDa.

Figure 5. Enitociclib is effective against MM *in vivo*. (A and B) *In vivo* mechanism of action of enitociclib in mice bearing JJN-3 MM xenografts upon a single dose of 15 mg/kg enitociclib administered intravenously compared to 80% PEG400 vehicle. Messenger RNA transcript (A) and (B) protein levels were normalized to housekeeping genes in L32 and β-actin, respectively. (*) $p < 0.05$. (C) *In vivo* antitumor activity of enitociclib administered as a single agent. For JJN-3, NCI-H929, and OPM-2 MM xenografts, enitociclib was dosed 15 mg/kg intravenously once weekly compared to vehicle control. (D) To study combinations, enitociclib was dosed 15 mg/kg intravenously once weekly in combination with 50 mg/kg lenalidomide orally daily or 0.8 mg/kg bortezomib intraperitoneally twice weekly in OPM-2 MM xenografts.

Figure 1

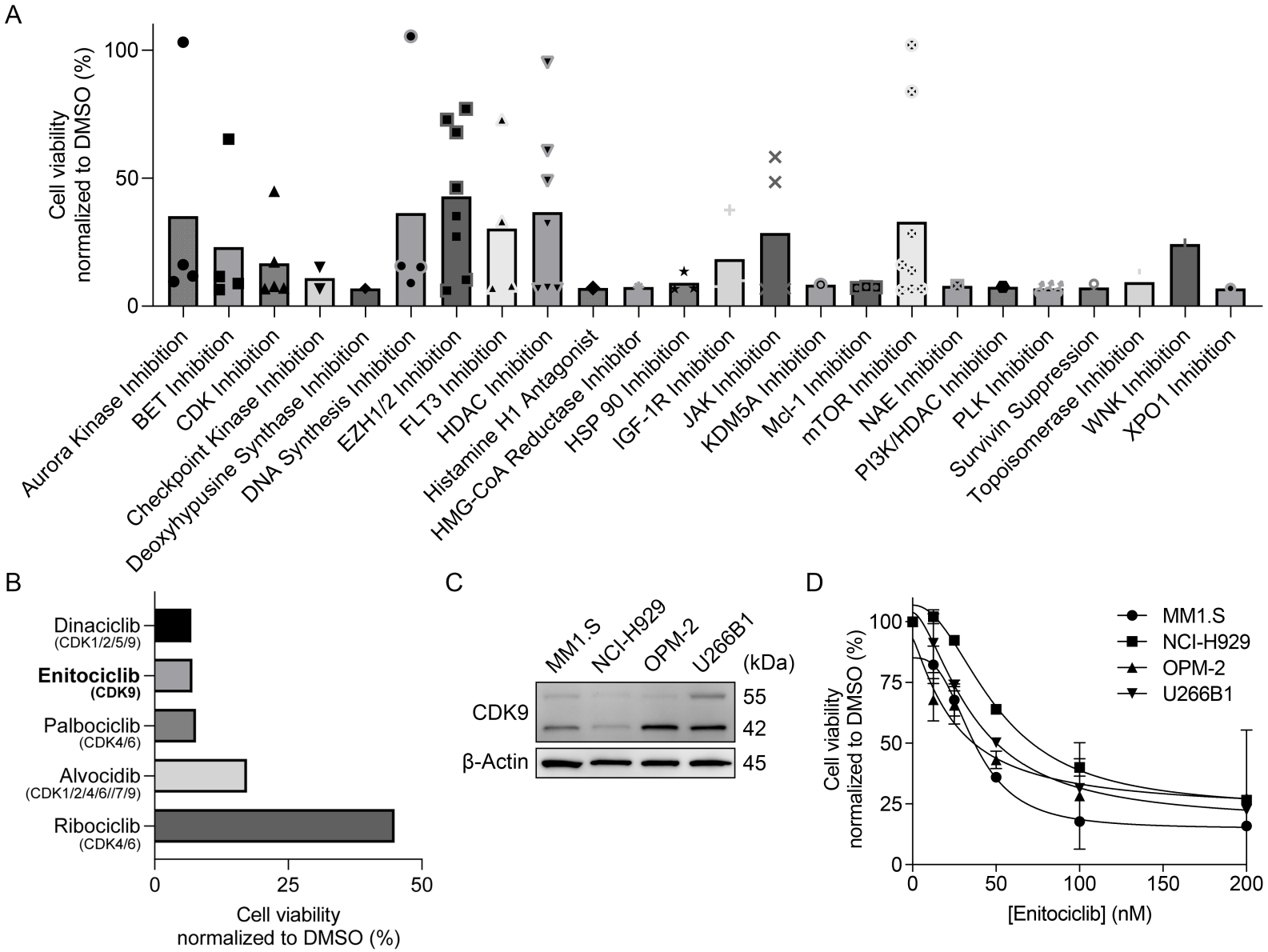

A
Cell viability
normalized to DMSO (%)
100
50
0
Aurora Kinase Inhibition
BET Inhibition
CDK Inhibition
Checkpoint Kinase Inhibition
Deoxyhypusine Synthase Inhibition
DNA Synthesis Inhibition
EZH1/2 Inhibition
FLT3 Inhibition
HDAC Inhibition
Histamine H1 Antagonist
HMG-CoA Reductase Inhibitor
HSP 90 Inhibition
IGF-1R Inhibition
JAK Inhibition
KDM5A Inhibition
Mcl-1 Inhibition
mTOR Inhibition
NAE Inhibition
PI3K/HDAC Inhibition
PLK Inhibition
Survivin Suppression
Topoisomerase Inhibition
WNK Inhibition
XPO1 Inhibition
B
Dinaciclib
(CDK1/2/5/9)
Enitociclib
(CDK9)
Palbociclib
(CDK4/6)
Alvocidib
(CDK1/2/4/6//7/9)
Ribociclib
(CDK4/6)
0
25
50
Cell viability
normalized to DMSO (%)
C
MM1.S
NCI-H929
OPM-2
U266B1
(kDa)
CDK9
55
42
β-Actin
45
D
Cell viability
normalized to DMSO (%)
100
75
50
25
0
0
50
100
150
200
[Enitociclib] (nM)
MM1.S
NCI-H929
OPM-2
U266B1

Figure 2

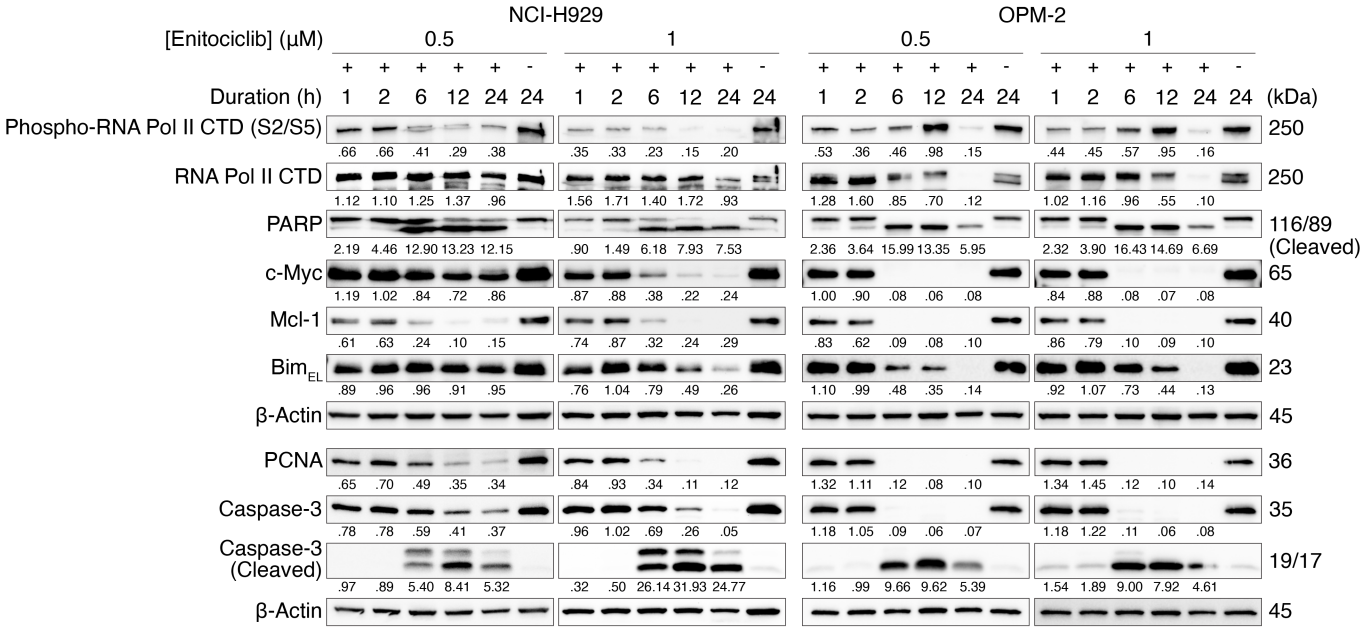

NCI-H929
OPM-2
[Enitociclib] (μM)
0.5
1
0.5
1
Duration (h)
1 2 6 12 24 24 1 2 6 12 24 24 1 2 6 12 24 24 1 2 6 12 24 24
(kDa)
Phospho-RNA Pol II CTD (S2/S5)
.66 .66 .41 .29 .38 .35 .33 .23 .15 .20 .53 .36 .46 .98 .15 .44 .45 .57 .95 .16
250
RNA Pol II CTD
1.12 1.10 1.25 1.37 .96 1.56 1.71 1.40 1.72 .93 1.28 1.60 .85 .70 .12 1.02 1.16 .96 .55 .10
250
PARP
2.19 4.46 12.90 13.23 12.15 .90 1.49 6.18 7.93 7.53 2.36 3.64 15.99 13.35 5.95 2.32 3.90 16.43 14.69 6.69
116/89 (Cleaved)
c-Myc
1.19 1.02 .84 .72 .86 .87 .88 .38 .22 .24 1.00 .90 .08 .06 .08 .84 .88 .08 .07 .08
65
Mcl-1
.61 .63 .24 .10 .15 .74 .87 .32 .24 .29 .83 .62 .09 .08 .10 .86 .79 .10 .09 .10
40
Bim$_{EL}$
.89 .96 .96 .91 .95 .76 1.04 .79 .49 .26 1.10 .99 .48 .35 .14 .92 1.07 .73 .44 .13
23
β-Actin
45
PCNA
.65 .70 .49 .35 .34 .84 .93 .34 .11 .12 1.32 1.11 .12 .08 .10 1.34 1.45 .12 .10 .14
36
Caspase-3
.78 .78 .59 .41 .37 .96 1.02 .69 .26 .05 1.18 1.05 .09 .06 .07 1.18 1.22 .11 .06 .08
35
Caspase-3 (Cleaved)
.97 .89 5.40 8.41 5.32 .32 .50 26.14 31.93 24.77 1.16 .99 9.66 9.62 5.39 1.54 1.89 9.00 7.92 4.61
19/17
β-Actin
45

Figure 3

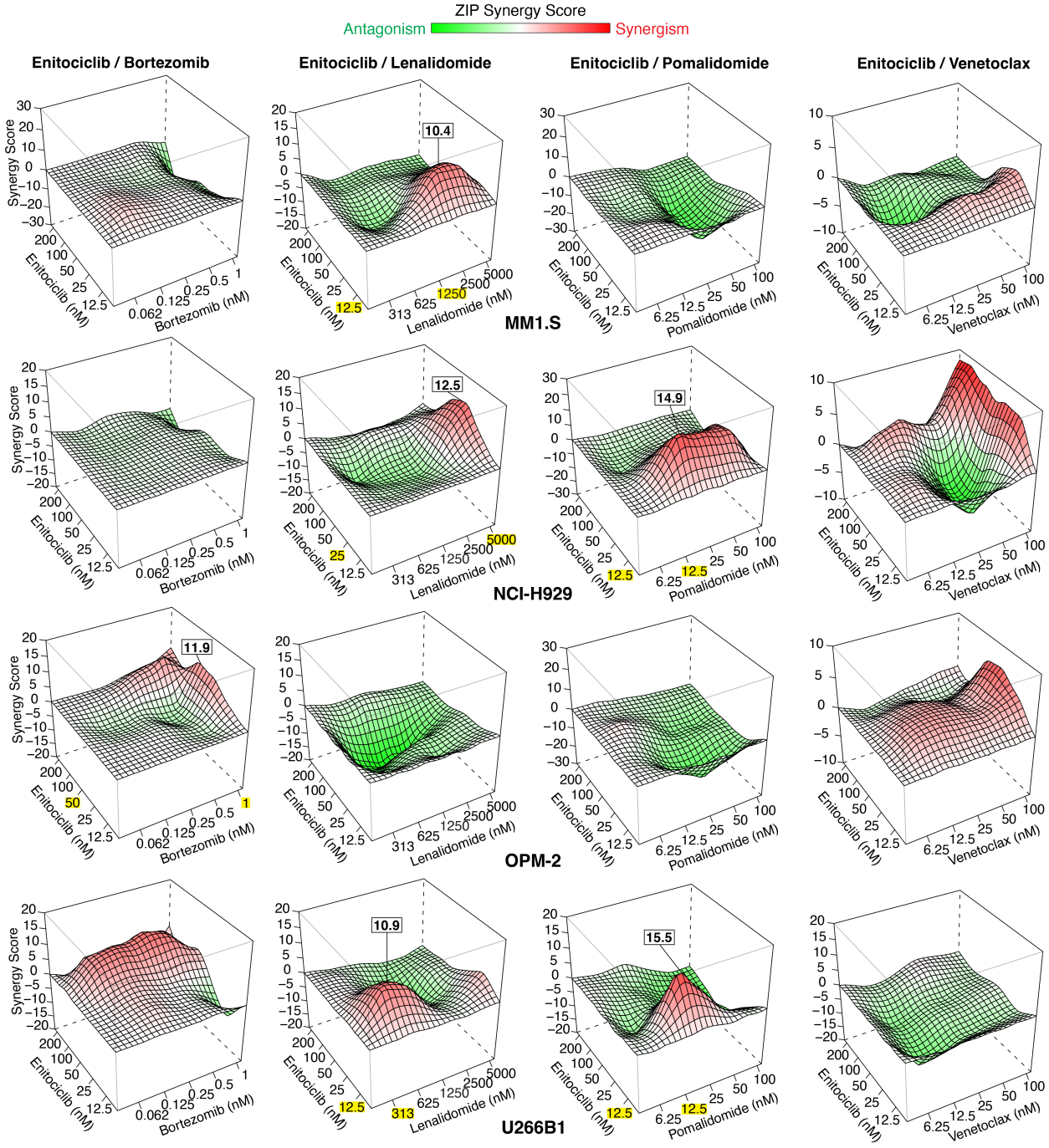

ZIP Synergy Score
Antagonism
Synergism
Enitociclib / Bortezomib
Enitociclib / Lenalidomide
Enitociclib / Pomalidomide
Enitociclib / Venetoclax
Synergy Score
Enitociclib (nM)
Bortezomib (nM)
Lenalidomide (nM)
Pomalidomide (nM)
Venetoclax (nM)
MM1.S
NCI-H929
OPM-2
U266B1
10.4
12.5
1250
12.5
14.9
5000
25
11.9
50
10.9
15.5

Figure 4

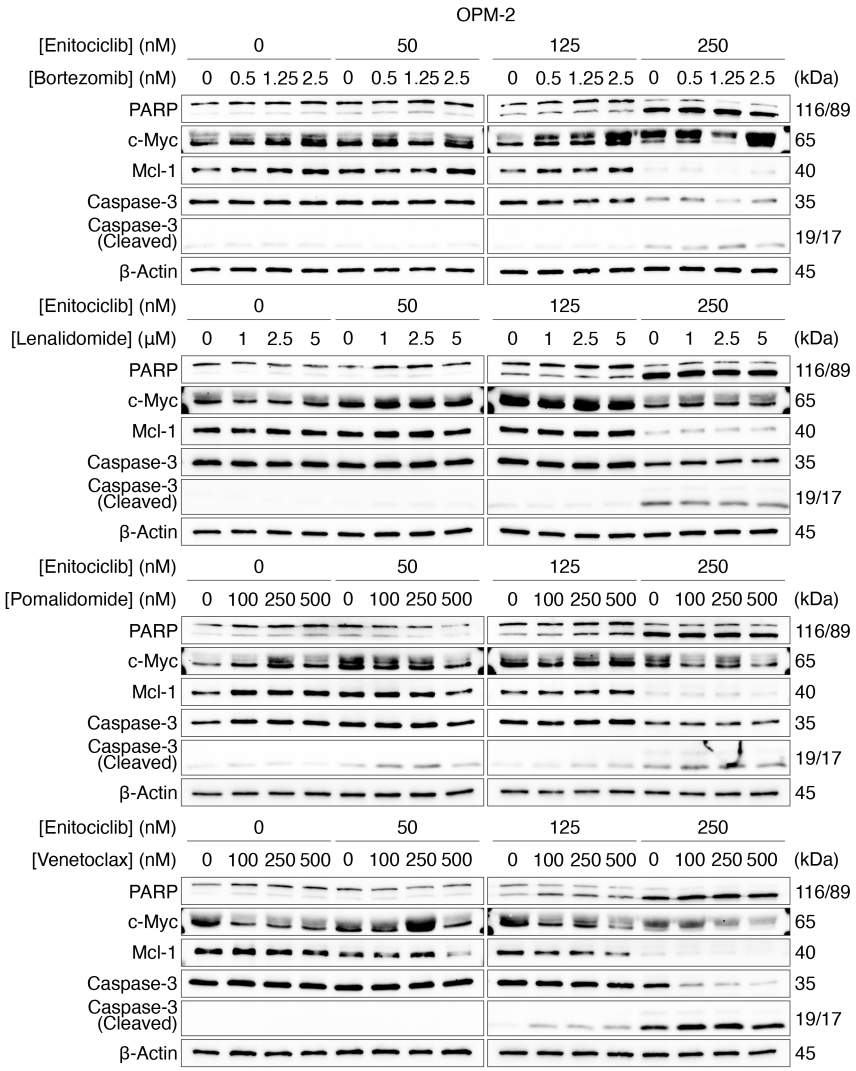
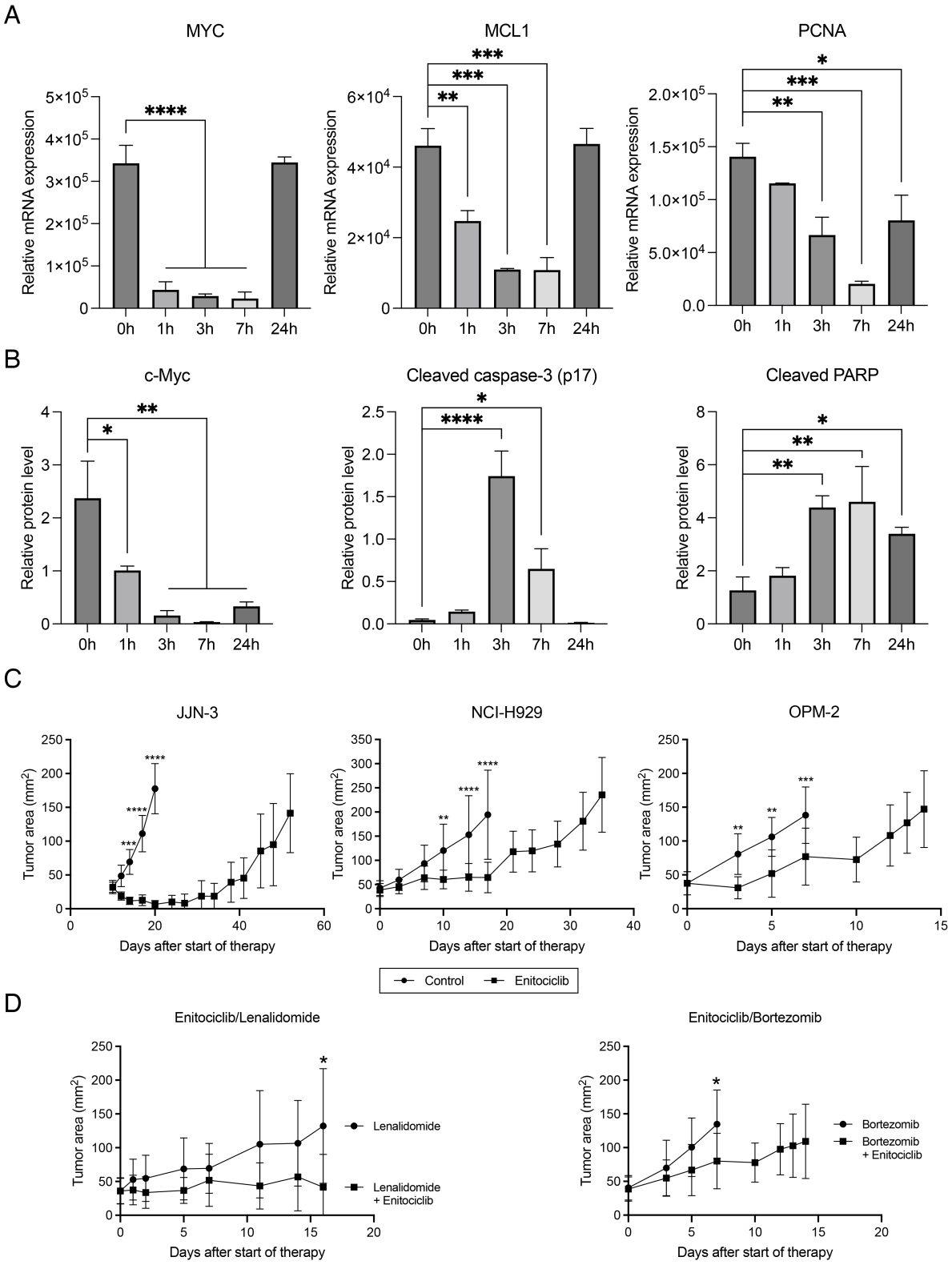

OPM-2
[Enitociclib] (nM)
0
50
125
250
[Bortezomib] (nM)
0 0.5 1.25 2.5 0 0.5 1.25 2.5 0 0.5 1.25 2.5 0 0.5 1.25 2.5
(kDa)
PARP
116/89
c-Myc
65
Mcl-1
40
Caspase-3
35
Caspase-3 (Cleaved)
19/17
β-Actin
45
[Enitociclib] (nM)
0
50
125
250
[Lenalidomide] (μM)
0 1 2.5 5 0 1 2.5 5 0 1 2.5 5 0 1 2.5 5
(kDa)
PARP
116/89
c-Myc
65
Mcl-1
40
Caspase-3
35
Caspase-3 (Cleaved)
19/17
β-Actin
45
[Enitociclib] (nM)
0
50
125
250
[Pomalidomide] (nM)
0 100 250 500 0 100 250 500 0 100 250 500 0 100 250 500
(kDa)
PARP
116/89
c-Myc
65
Mcl-1
40
Caspase-3
35
Caspase-3 (Cleaved)
19/17
β-Actin
45
[Enitociclib] (nM)
0
50
125
250
[Venetoclax] (nM)
0 100 250 500 0 100 250 500 0 100 250 500 0 100 250 500
(kDa)
PARP
116/89
c-Myc
65
Mcl-1
40
Caspase-3
35
Caspase-3 (Cleaved)
19/17
β-Actin
45

Figure 5


A
MYC
MCL1
PCNA
Relative mRNA expression
0h
1h
3h
7h
24h
B
c-Myc
Cleaved caspase-3 (p17)
Cleaved PARP
Relative protein level
C
JJN-3
NCI-H929
OPM-2
Tumor area (mm2)
Days after start of therapy
Control
Enitociclib
D
Enitociclib/Lenalidomide
Lenalidomide
Lenalidomide + Enitociclib
Enitociclib/Bortezomib
Bortezomib
Bortezomib + Enitociclib