

# The Novel Aurora Kinase Inhibitor ENMD-2076 Has Potent Activity against Multiple Myeloma (MM) *in Vitro* and *in Vivo*, and Shows Synergistic Activity in Combination with Lenalidomide

Xiaojing Wang<sup>1</sup>, Anthony L. Sinn<sup>2</sup>, Attaya Suvannasankha<sup>1</sup>, Colin D. Crean<sup>1</sup>, Li Chen<sup>1</sup>, Shuhong Zhang<sup>1</sup>, Jing Liang<sup>1</sup>, Genglin Zhang<sup>1</sup>, Karen E. Pollok<sup>2</sup>, Rafat Abonour<sup>1</sup>, Carolyn Sidor<sup>3</sup>, Mark R. Bray<sup>3</sup>, Sherif S. Farag<sup>1</sup>

<sup>1</sup>Department of Medicine, Division of Hematology and Oncology, Indiana University School of Medicine; <sup>2</sup>Indiana University Simon Cancer Center, *In Vivo* Therapeutics Core, Indianapolis, Indiana; and <sup>3</sup>EntreMed, Inc., Toronto, Ontario, Canada



## ABSTRACT

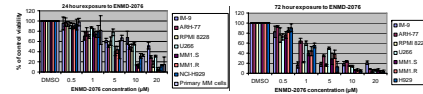
ENMD-2076 is a novel, orally-active molecule that has been shown to have significant activity against Aurora A kinase as well as multiple receptor tyrosine kinases (RTK). We investigated the single agent activity of ENMD-2076 against MM cells *in vitro* and *in vivo*, and in combination with lenalidomide. ENMD-2076 free base showed significant cytotoxicity against MM cells with a mean LC50 of 3.84±0.86 µM at 48 hours *in vitro*. Cytotoxicity was associated with cleavage of caspase 3, 8, 9 and PARP, and loss of mitochondrial membrane potential as early as 6 hours. ENMD-2076 free base inhibited downstream targets phosphorylated (p)-BAD, p-Foxo1a and p-GSK-3β at 6 hours and inhibition of Aurora kinases was observed at 24 hours. NOD/SCID mice implanted with H929 human plasmacytoma xenografts and treated for 30 days with 50, 100, 200mg/kg/d ENMD-2076 showed a dose-dependent inhibition of tumor growth, with minimal toxicity as assessed by the stable weight of treated animals. Immunohistochemical staining of tumors from sacrificed animals showed significant reduction in Ki67, p-H3 and CD34, and an increase in caspase-3 at all dose levels of treatment compared to control tumors. A reduction in p-FGFR-3 was observed on Western blot from the lysates of H929 tumors obtained from treated animals. ENMD-2076 free base also showed synergistic cytotoxic activity when combined with lenalidomide against H929, MM1.R and MM1.S cells as assessed by MTT assay and Annexin-V/PI staining. Using the Chou-Talalay method, the combination indices (CI) were < 1 for all three cell lines across a range of concentrations of ENMD-2076 free base (0.25-1.0 µM) plus lenalidomide (2.5-10 µM) indicating synergistic activity (CI=0.362 H929; CI=0.315 MM1.R; CI=0.415 MM1.S). Our results provide rationale for the investigation of ENMD-2076 alone and in combination with lenalidomide in patients with multiple myeloma.

## METHODS

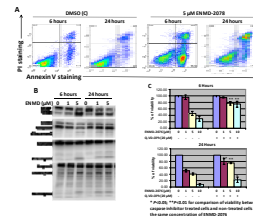
Cytotoxicity was assessed using MTT assay and annexin V/PI staining, and cell apoptosis and cell cycle were analyzed by flow cytometry, and intracellular signaling by Western blotting. Human plasmacytoma xenograft mouse model was set up in NOD-SCID mice using human MM cell line NCI-H929.

## RESULTS

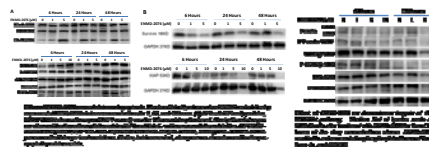
### 1. ENMD-2076 is cytotoxic to MM cells.



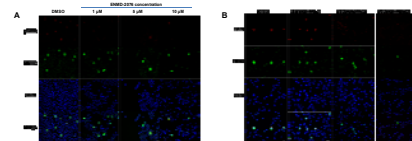
### 2. ENMD-2076 induces apoptosis of MM cells *in vitro* with activation of caspases.



### 3. ENMD-2076 cleaves Mcl-1 and downregulates pro-apoptotic proteins XIAP and survivin, and inhibits the PI3K/Akt pathway with downregulation of downstream effectors.

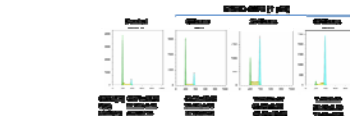


### 4. ENMD-2076 inhibits aurora kinases A and B, and induces G2/M cell cycle arrest in MM cells.



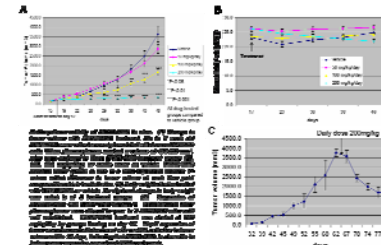
Immunofluorescence micrograph of MM1R cells treated with ENMD-2076 for 24 hours, showing inhibition of (A) aurora A, and (B) aurora B. p728 reflects staining (red) for phospho-aurora A in panel A, p-H3 reflects staining of p-H3 Ser10 in panel B. MPM2 reflects staining of cells in mitosis, and DAPI staining of DNA is a general marker of cells. The bottom row shows merge of the images of each of the stains to assist in localizing of the stain to particular cells. As shown, ENMD-2076 resulted in a reduction of p728 staining cells, indicating inhibition of aurora A.

## RESULTS

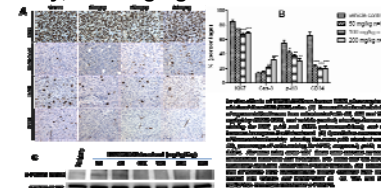


ENMD-2076 and lenalidomide combination assay. ENMD-2076 cells were treated with 2.5-10 µM ENMD-2076 free base and 2.5-10 µM lenalidomide for 72 hours. The percentage of cells in G2/M, G1/S, and G1/G0 phases were analyzed by flow cytometry. The percentage of cells in G2/M phase was significantly higher than the respective number of untreated cells, indicating that ENMD-2076 induces G2/M arrest.

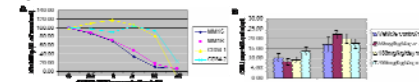
### 5. ENMD-2076 is active against MM cells *in vivo*.



### 6. ENMD-2076 inhibits proliferation, aurora kinases, ectopic FGFR-3 tyrosine kinase activity, and angiogenesis in MM cells *in vivo*.



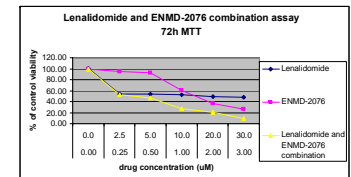
### 7. ENMD-2076 induces minimal toxicity to hematopoietic progenitors *in vitro* and *in vivo*.



ENMD-2076 does not induce hematopoietic progenitors. (A) Viability of purified human CD34+ cells treated with ENMD-2076. (B) Viability of purified human CD34+ cells treated with ENMD-2076. (C) Viability of purified human CD34+ cells treated with ENMD-2076. (D) Viability of purified human CD34+ cells treated with ENMD-2076. (E) Viability of purified human CD34+ cells treated with ENMD-2076. (F) Viability of purified human CD34+ cells treated with ENMD-2076. (G) Viability of purified human CD34+ cells treated with ENMD-2076. (H) Viability of purified human CD34+ cells treated with ENMD-2076. (I) Viability of purified human CD34+ cells treated with ENMD-2076. (J) Viability of purified human CD34+ cells treated with ENMD-2076. (K) Viability of purified human CD34+ cells treated with ENMD-2076. (L) Viability of purified human CD34+ cells treated with ENMD-2076. (M) Viability of purified human CD34+ cells treated with ENMD-2076. (N) Viability of purified human CD34+ cells treated with ENMD-2076. (O) Viability of purified human CD34+ cells treated with ENMD-2076. (P) Viability of purified human CD34+ cells treated with ENMD-2076. (Q) Viability of purified human CD34+ cells treated with ENMD-2076. (R) Viability of purified human CD34+ cells treated with ENMD-2076. (S) Viability of purified human CD34+ cells treated with ENMD-2076. (T) Viability of purified human CD34+ cells treated with ENMD-2076. (U) Viability of purified human CD34+ cells treated with ENMD-2076. (V) Viability of purified human CD34+ cells treated with ENMD-2076. (W) Viability of purified human CD34+ cells treated with ENMD-2076. (X) Viability of purified human CD34+ cells treated with ENMD-2076. (Y) Viability of purified human CD34+ cells treated with ENMD-2076. (Z) Viability of purified human CD34+ cells treated with ENMD-2076.

## RESULTS

### 8. ENMD-2076 shows synergistic cytotoxic activity against MM cell lines when combined with lenalidomide.



Using the Chou-Talalay method, the combination indices (CI) were < 1 for all three cell lines across a range of concentrations of ENMD-2076 free base (0.25-1.0 µM) plus lenalidomide (2.5-10µM) indicating synergistic activity.

Table 1. CIs generated from isobologram at different concentration of ENMD-2076 and lenalidomide

Cell line	ENMD-2076 (µM)	lenalidomide (µM)	Fractional activity	CI
H929	1	10	0.7276	0.362
MM1R	1	10	0.7455	0.315
MM1S	1	10	0.7184	0.404

## CONCLUSIONS

1. ENMD-2076 alone shows significant *in vitro* and *in vivo* antitumor activity against MM cells, and acts via multiple mechanisms.
2. ENMD-2076 induces minimal cytotoxicity to human and murine hematopoietic progenitors *in vitro* and *in vivo*.
3. ENMD-2076 shows synergistic cytotoxic activity when combined with lenalidomide against MM cells.
4. The results provide rationale for the investigation of ENMD-2076 alone and in combination with lenalidomide in patients with multiple myeloma.