

Abstract

ENMD-1198 (2-methoxyestra-1, 3, 5, (10), 16-tetraene-3-carboxamide), an analogue of 2-methoxyestradiol (2ME2 or Panzen®), is a microtubule destabilizing agent that binds to the colchicine-binding site of β -tubulin. ENMD-1198 has shown antiangiogenic and antiproliferative activity in several tumor models and is currently being evaluated in a Phase 1 clinical trial. To date however, the efficacy and mechanisms of action of ENMD-1198 in leukemia are not well-studied or fully understood. In order to assess the efficacy of ENMD-1198 in leukemia, a clinically relevant model of primary human ALL cells xenografted into immunodeficient (NOD/SCID) mice was used. Three human ALL xenografts (ALL3, ALL7 and ALL19) that exhibit intrinsic differences in response to vincristine (VCR) were treated with 100 mg/kg ENMD-1198 by gavage (daily for 28 days), commencing treatment when engraftment rates reached 1% human CD45⁺ in mouse peripheral blood. Treatment with ENMD-1198 significantly increased the mouse survival rates compared to vehicle control in all three xenografts (Leukemia Growth Delay (LGD) for ALL3 = 17.3 days, $p < 0.005$; ALL7 = 21.5 days, $p < 0.005$; ALL19 = 16.7 days, $p < 0.005$). Interestingly, ALL7, the least sensitive xenograft to vincristine, showed the best response to ENMD-1198 with a growth delay factor of 21 days. To determine whether the combination of ENMD-1198 and VCR has therapeutic advantages in leukemia, antiproliferative studies of the drug combination in CCRF-CEM leukemia cells were carried out. A synergistic effect was observed when ENMD-1198 and VCR were combined. The effect of this drug combination was further examined in the ALL human xenograft mouse model. Mice inoculated with ALL7 xenograft were treated with 50 mg/kg ENMD-1198 (daily for 28 days) and 0.5 mg/kg VCR (weekly for 4 weeks) by intraperitoneal injection. The drug combination significantly prolonged mouse survival rates compared to single ENMD-1198 and VCR treatments (LGD 35.19 days; $p < 0.005$). Functional analysis of the drug combination treatment in CCRF-CEM cells *in vitro* showed that the cells arrested at G₂/M followed by sub-G₁ (apoptosis) phase, with decreased Hif-1 α and JAK2 proteins. Apoptosis *in vitro* was associated with increased DR5, active caspase 3 and cleaved PARP proteins in CCRF-CEM cells treated with the combination. In summary, ENMD-1198 alone, and in combination with VCR, has shown promising results in the treatment of preclinical models of leukemia.

Results and Discussion

Drug sensitivity of ALL xenografts to ENMD-1198

The efficacy of ENMD-1198 in leukemia was assessed in a clinically relevant model of primary human ALL cells xenografted into immunodeficient (NOD/SCID) mice¹. Three human ALL xenografts (ALL3, ALL7 and ALL19) that exhibit intrinsic differences in response to vincristine (VCR)¹ were treated when engraftment rates reached 1% human CD45⁺ in mouse peripheral blood with 100 mg/kg ENMD-1198 by gavage (daily for 28 days). The proportion of human CD45⁺ cells in the peripheral blood was monitored throughout and following the course of treatment, and the event-free survival (EFS) was calculated from the initiation of treatment by Kaplan-Meier analysis¹. All the mice were sacrificed when the engraftment rates reached 25% human CD45⁺. Treatment with ENMD-1198 significantly increased the mouse survival rates compared to vehicle control in all three xenografts ($P < 0.005$) (Figure 1). Leukemia Growth Delay (LGD) was calculated by subtracting the median EFS of vehicle control from drug treated xenograft.

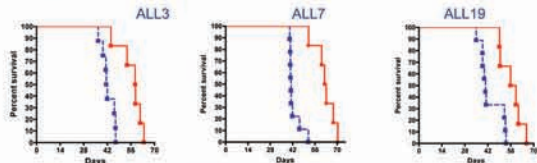


Figure 1: *In vivo* sensitivity of xenografts ALL3, ALL7 and ALL19 to ENMD-1198. Mice were inoculated with ALL3, ALL7 and ALL19, monitored for engraftment and treated with ENMD-1198 (■ solid lines) or vehicle control (● dotted lines). The EFS of NOD/SCID mice was quantified as the time taken from the initiation of treatment for the leukemic population to reach 25% in the mouse peripheral blood, or for the mice to show evidence of leukemic-related morbidity. Each line represents the proportion of mice remaining event free over time.

The leukemia growth delay (LGD) following ENMD-1198 treatment for ALL3, ALL7 and ALL19 were 17.3 days, 21.5 days and 16.7 days, respectively. Interestingly, ALL7, the least sensitive xenograft to VCR, showed the best response to ENMD-1198 with a growth delay factor of 21 days.

Effect of ENMD-1198 and VCR combination treatment *in vitro*

To investigate whether ENMD-1198 can be combined with VCR in ALL cytotoxicity assays (Alamar Blue™) and CalcuSyn analysis were used. Leukemia cells (CCRF-CEM) were treated with 1:1 combination ratio of ten different concentrations covering the IC₅₀ of both ENMD-1198 and VCR. The combination index (CI) plot is shown in Figure 2 where CI values at the range of combinations used were below 1. Overall, the results showed that the combination of ENMD-1198 and VCR is synergistic in CEM cells.

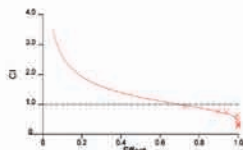


Figure 2: Combination index (CI) analyses of the effects of ENMD-1198 in combination with VCR in CEM cells. CI values were obtained from the mean of 3 independent experiments using CalcuSyn software. CI < 1 = synergistic; CI = 1 = additive; CI > 1 = antagonistic.

Effect of ENMD-1198 and VCR combination treatment of ALL7 xenograft

To determine if ENMD-1198 combined with VCR would increase the LGD compared to VCR alone, ALL7 xenograft was treated with 50 mg/kg ENMD-1198 (daily for 28 days) and 0.5 mg/kg VCR (once a week for 4 weeks). Combination treatment significantly prolonged mouse survival rates compared to single drug treatments (i.e. VCR alone and ENMD-1198 alone) in ALL7 xenografted mice (Figure 3). The LGD for combination treatment was 35.2 days (Table 1).

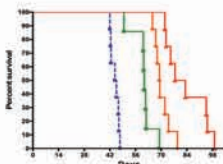


Figure 3: Mice were inoculated with ALL7 and treated with vehicle control (● dotted lines), ENMD-1198 (■ solid lines), VCR (▲ solid lines) or combination of ENMD-1198 and VCR (▲ solid lines).

Table 1: Response of ALL7 xenograft to combination treatment of ENMD-1198 and vincristine

Xenograft	Treatment	Median EFS (days)	LGD (days)
ALL-7	Vehicle control	45.14	-
	ENMD-1198	60.75	15.61**
	VCR	69.11	23.97**
	ENMD-1198 + VCR	80.33	35.19***

Leukemia Growth Delay (LGD) was calculated by subtracting the median EFS of vehicle control from drug treated xenograft. ** Significantly different from LGD vehicle control ($P < 0.005$). *** Significantly different from LGD single agent ENMD-1198 or vincristine ($P < 0.005$). Abbreviations: EFS, event free survival; VCR, vincristine.

Cell cycle analysis of CEM cells treated with ENMD-1198 and VCR combination

To investigate the potential mechanism(s) by which the combination of ENMD-1198 and VCR act synergistically *in vitro* and prolong leukemia growth *in vivo*, cell cycle analysis was performed. After 8 hours of drug exposure, combination treatment with ENMD-1198 and VCR greatly increased G₂/M cell cycle arrest compared to either drug alone (Figure 4). Moreover, following 24 hours drug exposure, there was an increase in the sub-G₁ (apoptosis) fraction in the drug combinations compared to either of the single drug treatments. Therefore, the combination of ENMD-1198 and VCR treatment in CEM cells *in vitro* enhanced mitotic arrest and apoptosis.

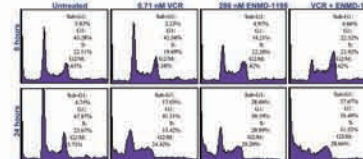


Figure 4: Cell cycle analysis of CEM cells after 8 and 24 hours of single or combination treatment of ENMD-1198 and VCR.

ENMD-1198 and vincristine in combination in the CCRF-CEM model increase apoptotic and decrease oncogenic proteins *in vitro* and *in vivo*

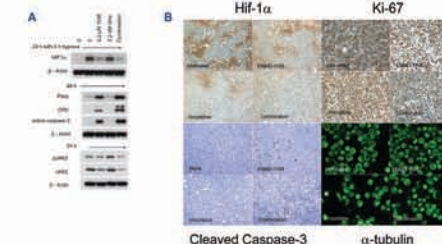


Figure 5: A. CCRF-CEM cells were treated with single agent (ENMD-1198 or vincristine) or combination as shown for 24 hours (Hif-1 α and pJAK2) or 48 hours (PARP, DR5, active caspase-3) and cells were lysed and analyzed by Western blotting. B. CCRF-CEM cells (10x10⁶) were implanted in matrigel in CB.17 SCID mice and treated with 6 doses ENMD-1198 (200 mg/kg), 2 doses vincristine (0.5 mg/kg) or the combination when tumor volumes were approximately 700 mm³. Tumors were sectioned and stained or, for microtubule analysis, were digested to single cells, spun onto slides and stained for α -tubulin.

Conclusions

1. ENMD-1198 is effective in delaying the growth of ALL xenografts *in vivo*.
2. Combination of ENMD-1198 and VCR treatments showed:
 - synergistic effects in leukemia cells *in vitro*
 - prolonged ALL7 xenograft survival *in vivo*
 - induced mitotic arrest and apoptosis

Reference

1. Liem et al. (2004) Blood, 103: 3905-3914.

Conflict of Interest:

P. Burke, X. Chen and T.M. LaVallee are employees of Entremed, Inc.