Introduction

Annamycin (Ann) is an anthracycline antibiotic that is not cross-resistant with doxorubicin (DOX), and formulated in liposomes. Liposomal annamycin (L-Ann) is currently in clinical studies in acute myeloid leukemia (AML) patients. In contrast to DOX, high levels of annamycin were observed to accumulate in multidrug resistant (MDR) cell lines, and consequently led to a significant degree of DNA damage and apoptosis. In vivo activity of L-Ann using alternative schedules was confirmed in aggressive AML mice models. In addition to high anticancer activity, in sharp contrast to DOX, free annamycin as well as L-Ann displayed dramatically reduced or no cardiotoxicity in preclinical toxicity studies.

Objective

The objective of the study was to assess the activity of Ann and its liposomal formulation (L-Ann) in clinically relevant models of AML.

Methods

• In vitro uptake of Ann in AML cell lines was assed using flow cytometry
• Subcellular distribution of Ann was determined by fluorescence microscopy using a Leica SP8 confocal system
• In vitro properties of Ann were assessed in human AML cell lines and included: a) histone H2AX phosphorylation at S139, b) induction of apoptosis by analysis of caspase 3 and PARP cleavage, and c) 72 h proliferation assays
• Single and multiple dose toxicity of Ann was assessed in healthy CD-1 or B6 mice
• In vivo evaluation of L-Ann was performed in: a) OCI-AML1 cells expressing Firefly luciferase in NOD SCID gamma (NSG) mice [1] b) an AML mouse model bearing the MLL/ENL-FLT3/ITD(p53−) mutations co-expressing high levels of the cyan fluorescent protein-mTurquoise2

Results

Ann is a lipophilic anthracycline antibiotic with properties superior to that of DOX. This novel liposomal formulation of Ann (L-Ann) was developed with entrapment efficiencies exceeding 80%. L-Ann displayed remarkable stability showing no degradation over 6 months [2]. Annamycin is a fluorescent compound with excitation and emission spectra at 490 and 576 nm, respectively. The uptake studies performed for MOLM-13, THP1 and MV4;11 cells show dose dependent accumulation of Ann in AML cells (Fig. 2A). Based on fluorescence intensity, the uptake of Ann was significantly higher than that of DOX in all tested cell lines. Fluorescence microscopy revealed a significantly different subcellular distribution of Ann when compared to DOX. In addition to nuclear localization, Ann accumulates to a high degree in the cytosol (Fig. 2B), which localizes predominantly in the nucleus.

Western blot analysis showed clear formation of DNA double strand breaks (DSBs) in OCI-AML3 cells exposed to Ann for 4 h and the extent of DSB was significantly higher than that observed for DOX. DSB induction triggered apoptosis manifested by caspase 3 and PARP cleavage (Fig. 2C). Proliferation assay studies of Ann determined that IC50 values were in the range from 1 nM to 100 nM (Fig. 2D).

Toxicity. Our previously published results of 14-days single dose toxicity of L-Ann in CD1 mice have shown an LD50 of 15.74 mg/kg [3]. In chronic exposure studies, L-Ann was remarkably less cardiotoxic than DOX. Cumulative toxicity with weekly administration of a given fraction of the subacute LD10 was markedly higher for DOX than for L-Ann as assessed by body weight and mortality studies. Multidose toxicity studies performed in B6 albino mice showed no deaths or body weight reduction in mice receiving L-Ann at 6 mg/kg (7 doses) and 4 mg/kg (12 doses). Animals receiving L-Ann at 8 mg/kg showed significant weight loss and died in the third injected schedule (Fig. 3A). Dose – dependent reduction of the white blood cell count was observed one week after injection of the first dose (Fig. 3B).

Efficacy of L-Ann in human model of AML. In vivo efficacy of L-Ann was tested in a human model of AML in NSG mice. Figure 4 shows the results of bioluminescent imaging twenty days after tumor inoculation. L-Ann treated mice showed significant delays of tumor growth after one or two doses of L-Ann. Despite the remarkable response, hypersensitivity of NSG mice to anthracyclines does not allow for more extensive studies of L-Ann scheduling modifications in this model.

Efficacy of L-Ann in murine AML model. We assessed the efficacy of L-Ann in a novel AML model that allows visualizing the dynamics of individual AML cells in vivo by two-photon microscopy. In this model, mouse AML cells bearing the MLL/ENL-FLT3/ITD(p53−) mutations co-express high levels of the cyan fluorescent protein-mTurquoise2. Upon intravenous infusion of 1 x 10^7 AML-Turq2 cells into syngeneic immunocompetent BALB/c mice, initial AML disease reliably develops within 2 weeks. Figure 5 is an example of peripheral blood analysis of (A) vehicle and (B) L-Ann-treated mice on day 11 after tumor cell injection and (C) corresponding survival curves. The median survival of L-Ann treated mice was 31 days and was more than twice as long as for mice receiving vehicle (p = 0.0006). Two-long term surviving mice were identified in a group treated with L-Ann at 4 mg/kg.

Efficacy of L-Ann in a murine AML model with delayed initiation treatment. In this study, mice injected with AML-Turq2 cells received five different doses of L-Ann on day 4 and 11. Analysis of peripheral blood showed dose-dependent delay in AML progression (Fig. 6A) and significant extension of survival for doses of 1 mg/kg of L-Ann and above (Fig. 6B). To further investigate the efficacy of L-Ann in a more delayed treatment was delayed until the blast levels in circulation reached 1-2% (day 10 after tumor cell injection – 4 days control group started to die). L-Ann at 4 mg/kg was given once weekly for 4 weeks. Survival analysis shows a dramatic extension of bioluminescent tumor survival of 37 days (p = 0.0002) (Fig. 6C).

Assessment of L-Ann anti-AML efficacy in specific organs. To investigate the tissue organ distribution of AML cells before and after L-Ann treatment, the mice were dosed with the drug ten days after inoculation of tumor cells. The imaging performed three days after dosing (Fig. 7) revealed a significant reduction of AML cells in bone marrow and spleen after single dose. Remarkably, L-Ann treatment eradicated AML cells in lungs, liver and lymph nodes of the treated mice (Fig. 7).

Summary

• Annamycin is a potent anti-leukemic agent with IC50 ranging from 1 to 100 nM in AML cell lines tested
• L-Ann shows dose and time-dependent accumulation in cells increased cytosolic localization
• L-Ann is well tolerated in vivo – multiple weekly doses in mice at 4 and 6 mg/kg doesn’t result in weight loss or mortality
• L-Ann reduces blasts in an OCI-AML3 model after a single dose of 4 mg/kg
• L-Ann displays a dose-dependent inhibition of blasts detected in the peripheral blood and significantly improves survival in an extremely aggressive murine AML-Turq2 model
• L-months study indicates AML cells in lungs, liver and lymph nodes of treated mice and caused a significant reduction of the blasts in bone marrow and spleen
• Multiple weekly administration of L-Ann (4 mg/kg) in mice models appears to be a novel, promising and safe treatment schedule

Acknowledgments and Disclosures

We thank Dr. Peter Ruttke for sharing THP1, MV4-11 and OCI-AML3 cell lines and help with OCI-AML3 NSG model. We thank Dr. Liu Chen for sharing the Moln 13 cell line

This work was supported by grant from Molecular Biotech, Inc. Dr. Waldemar Priebe is Chairman of the Scientific Advisory Board and owns stock in Molecular Biotech, Inc. (NASDAQ: MBRX).

References

