An orally bioavailable parthenolide analog selectively eradicates acute myelogenous leukemia stem and progenitor cells

Running Title: A parthenolide analog targets leukemia stem cells

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Abstract

Leukemia stem cells (LSCs) are thought to play a central role in the pathogenesis of acute leukemia and likely contribute to both disease initiation and relapse. Therefore, identification of agents that target LSCs is an important consideration for the development of new therapies. To this end, we have previously demonstrated that the naturally-occurring compound parthenolide (PTL) can induce death of human LSCs in vitro, while sparing normal hematopoietic cells. However, PTL has relatively poor pharmacological properties that limit its potential clinical use. Consequently, we generated a family of PTL analogs designed to improve solubility and bioavailability. These studies identified an analog, dimethylamino-parthenolide (DMAPT), which induces rapid death of primary human LSC from both myeloid and lymphoid leukemias, and is also highly cytotoxic to bulk leukemic cell populations. Molecular studies indicate the prevalent activities of DMAPT include induction of oxidative stress responses, inhibition of NF-kB, and activation of p53. The compound has approximately 70% oral bioavailability and pharmacological studies using both mouse xenograft models and spontaneous acute canine leukemias demonstrate in vivo bioactivity as determined by functional assays and multiple biomarkers. Therefore, based on the collective preclinical data, we propose that the novel compound DMAPT has the potential to target human LSCs in vivo.
Introduction

Recent studies have demonstrated that myeloid and certain forms of lymphoid leukemia arise from malignant stem cells (termed leukemia stem cells, LSCs). LSCs are typically found in a quiescent state and are thus unlikely to respond to standard chemotherapeutic agents which preferentially eradicate actively cycling cells. Indeed, the persistence of LSCs following chemotherapy may be a major factor contributing to clinical relapse. In addition, conventional leukemia therapy is also substantially toxic to normal hematopoietic cells and frequently results in severe myelosuppression. Therefore, given the drug refractory nature of LSCs, and the importance of normal hematopoiesis, identification of less toxic and more specific forms of therapy are important priorities for the development of better therapeutic regimens.

As a foundation for developing more selective leukemia treatments, our previous experiments have investigated basic properties of primitive AML cells. These studies showed that LSCs from different AML subtypes share characteristics which are unique to AML and thus represent potential therapeutic targets for the selective ablation of LSCs relative to their normal counterparts. Specifically, we reported that NF-κB, a known regulator of growth and survival, is constitutively active in LSCs but not in normal hematopoietic stem cells (HSCs). Notably, many traditional cancer therapies induce activation of NF-κB, a potentially undesirable characteristic likely to facilitate survival of malignant cells. Given the ability of many cancer cells to evade apoptosis, we hypothesized that NF-κB inhibition could be used to facilitate LSC-selective cell death, a concept supported by studies using the proteasome inhibitor MG-132 (known to inhibit NF-κB) with the anthracycline Idarubicin (IDR). However, molecular genetic approaches demonstrated that NF-κB inhibition alone is not sufficient to strongly induce AML-specific apoptosis. Further investigation of pathways induced by MG-132 + IDR treatment revealed activation of p53 and increased oxidative load as prevalent components of the AML cell death process. Collectively, these data suggest that the mechanism of LSC death involves combined inhibition of survival pathways and activation of tumor suppressor and/or stress pathways. More recently, we have
shown that robust apoptosis of primary AML cells can be achieved with a single agent, the plant derived compound parthenolide (PTL), which is known to induce oxidative stress and inhibit NF-κB. Importantly, PTL also effectively eradicates AML stem and progenitor cells \textit{in vitro} while sparing normal hematopoietic cells. Hence, PTL has the ability to eradicate AML stem cells, as well as to ablate bulk leukemia blast cells; properties that should make this compound an attractive agent for clinical evaluation. However, despite the utility of PTL determined by in vitro studies, it’s solubility is relatively poor, making pharmacological use of the compound difficult. In animal studies, maximum attainable serum levels were 200nM, a concentration approximately 30 fold less than required to mediate LSC cell death \textit{in vitro}. Therefore, we have synthesized and screened PTL analogs to identify a compound with improved solubility and bioavailability. These studies have generated a dimethylamino analog of parthenolide (DMAPT). When formulated as a fumarate salt DMAPT demonstrates over 1000 fold greater solubility in water relative to PTL (Neelakantan et al., manuscript in preparation). Moreover, as shown in the present studies, DMAPT effectively eliminates human AML stem and progenitor cells without apparent harm to normal hematopoietic stem and progenitor cells. The compound also eradicates phenotypically primitive blast crisis CML and acute lymphoblastic leukemia (ALL) cells. The molecular responses to DMAPT, both \textit{in vitro} and \textit{in vivo}, include activation of cellular stress responses and inhibition of NF-κB. Together, the data suggest that DMAPT represents a clinical candidate for leukemia therapy with the potential to target leukemia stem and progenitor cells.

\section*{Materials and Methods}

\subsection*{Cell Isolation and Culture}
Primary human AML, CML, ALL cells, and normal bone marrow (BM) cells were obtained from volunteer donors with informed consent. All manipulation and analysis of human specimens was approved by the University of Rochester Institutional Review Board. Umbilical cord blood (CB) was obtained from the National Disease Research Interchange (NDRI). Dog samples were obtained from Bellingham Veterinary Clinic,
Colorado State University (Department of Pathology) or Redbank Veterinary Hospital (Case Study II). Mononuclear cells were isolated from the samples using Ficoll-Plaque (Pharmacia Biotech, Piscataway, NY) density gradient separation. For canine case studies WBC numbers were determined using The HESKA® CBC-Diff™ System. In some cases cells were cryopreserved in freezing medium of Iscove’s modified Dulbecco medium (IMDM), 40% fetal bovine serum (FBS), and 10% dimethylsulfoxide (DMSO) or in CryoStor™ CS-10 (VWR). Cells were cultured in serum-free medium (SFM) for 1h before the addition of DMAPT. Parthenolide was obtained from Biomol (Plymouth Meeting, PA).

**DMAPT synthesis and pharmacology**

DMAPT was prepared from the reaction of parthenolide with dimethylamine, and the resulting dimethylamino analog was then converted to its water-soluble fumarate salt. The detailed synthesis, structural identity and stereochemistry of DMAPT are reported elsewhere (Neelakantan et al., manuscript in preparation). Bioavailability and pharmacokinetic assays for rodents were performed by the Developmental Therapeutics Program from the National Cancer Institute and for dogs by Integrated Analytical Solutions, Berkeley, CA.

**Methylcellulose Colony-Forming Assay**

AML, BM or CB cells were cultured in SFM as above for 18h in the presence or absence of DMAPT. Cells were plated at 50,000 cells/ml in Methocult GFH4534 (Stem Cell Technologies, Vancouver) supplemented with Erythropoietin 3U/ml and G-CSF 50ng/ml. Colonies were scored after 10-14 days of culture.

**Electrophoretic Mobility Shift Assay (EMSA), and Immunoblot Analysis**

EMSA was performed as described. Briefly, nuclear extracts equivalent to 200,000 cells were incubated with 2µg of poly-d(l-C) (Roche Molecular Biochemicals, Indianapolis, IN) and 10⁻¹⁴mol ³²P-labeled NF-κB probe in 10mM HEPES, 5mM Tris, 50mM KCl, 1.2mM EDTA and 10% glycerol for 15 minutes at room temperature. Protein/DNA complexes were resolved on a native polyacrylamide gel in 0.25X TBE.
For immunoblots, cells were prepared and analyzed as previously described. Blots were probed with anti-phospho-p53 (ser15) from Cell Signaling (Beverly, MA), and anti-actin (AC-15) from Sigma (St. Louis, MO).

**Confocal microscopy**

Cells were fixed in methanol at -20°C. The cells were permeabilized with blocking buffer (10% FBS and 0.1% Tween 20 in 1x PBS [pH 7.4]) as described. Cells were stained using either rabbit polyclonal anti-p65 (C-20), anti-Nrf-2 (C-20) (Santa Cruz Biotechnologies, Santa Cruz, CA), anti-HO1 (GeneTex Inc., San Antonio, TX) or mouse monoclonal anti-γH2AX (Upstate Biotech, Charlottesville, VA) in blocking buffer for 2 hours at room temperature. Cells were washed and stained with either goat-anti-rabbit Alexa488 or goat-anti-mouse Alexa 488 (Invitrogen, Carlsbad, CA) secondary antibodies and ToPro3 for nuclear stain (Invitrogen). Slides were mounted using Fluoromount-G (Southern Biotech, Birmingham, AL). Slides were left to dry overnight. Fluorescence was observed using a 100x objective, further magnified by a 2x zoom, on a Leica inverted scanning confocal microscope.

**Flow Cytometry**

Apoptosis assays were performed as described. Briefly, after 18-24h of treatment, normal and AML specimens were stained for the surface antibodies CD38-allophycocyanin (APC), CD34-PECy7, CD123-phycoerythin (Becton Dickinson, San Jose, CA) for 15 minutes. Cells were washed in cold PBS and resuspended in 200µl of annexin-V buffer (0.01M HEPES/NaOH, 0.14M NaCl, 2.5mM CaCl2) Annexin-V-fluorescein isothiocyanate (FITC) and 7-aminoactinomycin (7-AAD; Molecular Probes, Eugene, OR) were added and the tubes were incubated at room temperature for 15 minutes then analyzed on a BD LSRII flow cytometer. Analyses for phenotypically described stem cell subpopulations were performed by gating CD34+/CD38-populations. To analyze human cell engraftment in the NOD/SCID xenotransplant model, BM cells were blocked with the anti-Fc receptor antibody 2.4G2 and 25% human serum and later labeled with anti-human CD45-PE antibody (Becton Dickinson, San Jose, CA). For canine studies cells were stained with CD45-FITC (YKIX716.13;
Serotec, Raleigh, NC), CD14-PeCy5 (TUK4; Serotec), CD34-PE (1H6; Becton Dickinson) (Serotec) for 30 minutes. Cells were washed and resuspended in FACS buffer (0.5% FBS in PBS) with 5 μg/ml of DAPI. To analyze canine cell engraftment in the NOD/SCID xenotransplant model, cells were labeled with anti-canine CD45-APC antibody (Serotec, Raleigh, NC) and anti-mouse CD45-FITC (Pharmingen) and anti-canine CD34. Cells were washed and resuspended in FACS buffer (0.5% FBS in PBS) with 5 μg/ml of DAPI.

Non-obese Diabetic (NOD)/Severe Combined Immunodeficient (SCID) Mouse Assays

NOD/SCID mice were sub-lethally irradiated with 270 rad using a RadSource-2000 x-ray irradiator before transplantation. Cells to be assayed were injected via tail vein in a final volume of 0.2ml of PBS with 0.5% FBS. For analysis of human cells, 5-10 million primary umbilical cord blood specimens were used for each recipient animal. For analysis of canine cells, 5 million peripheral blood leukocytes were used for each recipient animal. For secondary transplantation studies of canine cells, total marrow from primary NOD/SCID mice was harvested, analyzed for canine cell content (by FACS), and then immediately retransplanted into irradiated secondary recipients at a cell dose adjusted to contain 3 million canine cells. After 6-8 weeks, animals were sacrificed, and BM was analyzed for the presence of human or canine cells by flow cytometry.

Canine studies

In vitro and in vivo studies were performed with owner’s consent on three animals with a diagnosis of CD34-positive acute leukemia. Case study I – the subject was an 8 year old male Labrador retriever. At the initiation of treatment the animal was in advanced stages of disease and received anti-inflammatory agents, sedatives, diuretics, antibiotics, and prokinetic agents as needed during the course of DMAPT treatments. In addition, the dog received 40 mg of Prednisone starting at day 5, reduced to 20 mg on day 11 and Mesna 500 mg (3 x day) starting on day 8. At day 14 the animal succumbed to multiple symptoms associated with advanced disease. Case study II –
the subject was an 8 year old male mixed breed dog. At the beginning of treatment the WBC was highly elevated (81K/ul) and the animal was receiving Deramaxx 100mg SID. At day 15, the animal also began receiving Clavamox and Prednisone. At day 24 the animal was withdrawn from study and euthanized at the owner’s request due to concern there was not sufficient likelihood of cure to warrant continued treatment. Case study III – the subject was a 12 year old male Pug. Prior to the beginning of DMAPT treatment the WBC was 96.5K/µl and the animal was receiving Predisone (5mg), Famotidine, Clavamox and Hydrocodone. At day 14 the animal was withdrawn from study and euthanized at the owner’s request due to concern there was not sufficient likelihood of cure to warrant continued treatment.

**U937 differentiation assay**
U937 cells were plated at 400,000 cells/ml and treated with DMAPT (2.5µM or 5µM) or 5µM ATRA. Cells were counted and analyzed 72 hours after treatment for expression of CD 11b and viability (DAPI).

**Statistical analysis**
Statistical analyses and graphs were performed using GraphPad Prism software (GraphPad Software, San Diego, CA). For statistical analysis the data was log transformed and analyzed by one-way ANOVA followed by Tukey post-hoc test. For 2 group comparisons, significance was determined by two-tailed t-tests.

**Results**

**DMAPT selectively eradicates primitive leukemia cells**
DMAPT was prepared from the reaction of PTL with dimethylamine, and the resulting dimethylamino analog was then converted to its water-soluble fumarate salt (Neelakantan et al., manuscript in preparation). Initially, we performed detailed biological studies to determine the efficiency and specificity of anti-leukemia properties for DMAPT. Figure 1A shows that 24-hour exposure of primary human AML cells to either 7.5 µM PTL or DMAPT results in similar mean viability in total and CD34+ cell
populations (25% vs. 24%, and 12% vs. 12% respectively, n=25 for PTL and n=39 for DMAPT). Supplemental table 1 shows the percent viability for each of the primary AML specimens after treatment with either PTL or DMAPT. We further investigated the levels of cell death in phenotypically primitive AML stem cells (CD34+CD38-CD123+). Figure 1B shows the results of cells treated with DMAPT where 5.0µM and 7.5µM concentrations resulted in 15.10% (n=19) and 6.84% (n=21) mean viability respectively. Dose response studies show that the LD₅₀ for DMAPT in primary AML cells is 1.7 µM. Taken together, the data in Figures 1A and 1B indicate that DMAPT is highly cytotoxic to both overall AML blast cells, as well as the AML stem cell population. To extend our analyses of DMAPT, we also examined other hematological disorders known to derive from malignant stem cells. Supplemental table 2 provides the percent viability for primary blast crisis (bc) CML and B-ALL specimens after treatment with either PTL or DMAPT. Figure 1C and 1D show the effect of DMAPT on phenotypically described B-ALL stem/progenitor cells (CD34+CD10-) and CML stem/progenitor cells (CD34+CD38-). These experiments indicate DMAPT also has utility for lymphoid and chronic myeloid forms of leukemia. Finally, to verify the specificity of DMAPT for malignant cells, viability of normal hematopoietic cells was determined. Supplemental table 3 shows the percent viability for normal mononuclear cells obtained from healthy donors after treatment with either PTL or DMAPT. The data in Figure 1E demonstrate that DMAPT does not significantly affect viability of normal CD34+ or CD34+CD38-hematopoietic cells obtained from healthy donors (n=8; p >0.05). Indeed, the mean viability at 5µM DMAPT was 96% for CD34+ cells and 88% for CD34+CD38- cells. At 7.5µM, the mean viability for both CD34+ and CD34+CD38- populations was over 79%. Together, the data demonstrate that DMAPT not only induces rapid cell death in phenotypically described AML, CML and ALL stem/progenitor cells, but is also well tolerated by normal stem and progenitor cells.

**Functional assays demonstrate that DMAPT ablates primary human AML stem and progenitor cells**

*In vitro* colony assays and NOD/SCID xenotransplant experiments were used to determine whether DMAPT targets functionally defined leukemia progenitor and stem
Treatment of normal hematopoietic cells with 5µM DMAPT did not affect myeloid or erythroid colony formation relative to untreated controls (Figure 2A. P > 0.05, n=5). In contrast, DMAPT treatment strongly inhibited the ability of AML cells to form colonies (mean viability= 6.58%, P < 0.001, n=10), indicating selective targeting of leukemic progenitor cell populations. Similarly, 18-hour treatment of primary AML cells with DMAPT dramatically inhibited engraftment of sub-lethally irradiated NOD/SCID mice (representative example shown in Figure 2B). Analysis of four independent AML specimens decreased engraftment by 98.2% (n=5 mice, p<0.0001), 91% (n=5, p=0.001), 90% (n=9, p<0.002) and 85% (n=4, p<0.0001) compared to untreated controls. In contrast, of three independent normal specimens tested, engraftment levels for DMAPT treated cells were 144% (n=5, p=0.178), 166% (n=5 mice, p=0.77), and 65% (n=5, p=0.06) relative to untreated controls, with no changes reaching statistical significance. Together, these data indicate that DMAPT specifically ablates AML stem and progenitor cells without affecting the growth or engraftment potential of normal primitive cells.

**DMAPT treatment induces stress responses, inhibits NF-κB, and activates p53**

We have previously shown that induction of oxidative stress, inhibition of NF-κB, and activation of p53 are functions associated with anti-LSC activity in primary AML cells. In addition, we have identified prevalent pathways and genes affected by PTL using global gene expression analysis of CD34+ primary AML specimens (Hassane et al, manuscript in preparation). These experiments have identified strong up-regulation of NF-E2-related factor 2 (Nrf-2) and its transcriptional target heme oxygenase 1 (HO-1) in response to PTL treatment. Both genes are part of a cytoprotective response against oxidative stress, and provide potential biomarkers for PTL-based drug responses. To evaluate the molecular consequences of DMAPT treatment, we first examined changes in oxidative state using the free thiol reactive dye mBBr. As shown in Figure 3A, labeling with mBBr was consistently reduced in specimens treated with DMAPT, indicating a strong decrease in intracellular free thiol groups. Subsequent analysis of stress response mechanisms showed that HO-1 levels strongly increase after 2 hours of treatment (Figure 3B), indicating that DMAPT induces a protective response against...
oxidative stress in the cell (n=3). An induction of Nrf2 nuclear localization was also observed (data not shown). Interestingly, inhibition of NF-κB occurs more slowly, with some decrease evident within an hour, but maximal reduction is not apparent until 4-8 hours (Figure 3C, lower panel). Similarly, activation of p53, as detected by phosphorylation at ser15, is not evident until after approximately 8 hours of treatment (Figure 3C, top panel). Notably, the anti-leukemia effect of DMAPT on AML cells becomes non-reversible only after 8 or more hours of exposure (data not shown), suggesting that changes in NF-κB and p53 may represent final (or late) steps in the commitment to cell death process. Together, these data indicate that DMAPT induces a rapid induction of oxidative stress followed by a series of cellular responses that include downstream stress control proteins and modulation of both survival and tumor suppressor mechanisms. These findings are in good agreement with previous hypotheses on the mechanisms that regulate selective targeting of LSC.10

**DMAPT Pharmacology**

To further characterize the potential utility of DMAPT, preliminary pharmacological studies were performed. Not surprisingly, substantial pharmacokinetic (PK) differences were observed in rodent and canine studies. In mice, an oral DMAPT dose of 100mg/kg achieved a Cmax of 25 μM and a half-life (T1/2) of 0.63 hours in serum. In contrast, canine studies showed a Cmax of 61 μM, with a T1/2 of 1.9 hours when DMAPT was dosed at 100 mg/kg p.o. For both the mouse and canine models, oral bioavailability was approximately 70% in comparison to intravenous administration. These characteristics represent a significant improvement over PTL, which demonstrated a maximum serum concentration of 200 nM in mice when dosed at 40 mg/kg, which is the highest dose attainable given the relative insolubility of PTL.18 Furthermore, in preliminary toxicology studies, daily administration of 100 mg/kg DMAPT to mice for 10 consecutive days was well tolerated with no evidence of acute toxicity or changes in hematologic parameters. Similarly, daily oral dosing of dogs at 50-100 mg/kg for 14 consecutive days was well tolerated. Collectively, these studies indicate that the pharmacological properties of DMAPT are superior to PTL.
**In vivo biological activity of DMAPT in a murine model**

Increased oxidative stress and inhibition of NF-κB are consistent in vitro features of DMAPT treatment (Figure 3 panels A, B and C). Thus, we tested whether such responses were also evident in vivo where they could serve as potential biomarkers for DMAPT activity. To this end, we injected primary human AML cells into sub-lethally irradiated NOD/SCID mice to establish xenografts. At 6 weeks post-injection (a time at which the AML cells have strongly engrafted in bone marrow), mice were treated with a single oral dose of 100mg/kg DMAPT. One hour post-treatment, animals were sacrificed to evaluate the bioactivity of DMAPT in human AML cells isolated from the bone marrow. Figure 3D shows that the NF-κB p65 subunit (yellow) is localized to the cytoplasm upon drug treatment (nucleus shown in blue), an activity indicating substantial inhibition of NF-κB (n=3). In addition, figure 3E shows that Nrf2 (green) localized to the nucleus, indicating its typical activation in response to oxidative stress (n=3). Together, these data indicate that DMAPT induces both the activation of stress responses and inhibition of NF-κB in vivo, and that both of these measurements can be used as biomarkers to monitor drug activity.

**In vivo biological activity of DMAPT in spontaneous canine leukemias**

Spontaneous leukemias are well documented in dogs, and thus provide a large animal system in which to investigate drug activities. In addition, as mentioned above, the pharmacology observed for DMAPT was notably better in dogs than in mice. Therefore, we employed studies of primary canine leukemia as a means to further characterize the activity of DMAPT. First, in vitro studies were conducted to examine whether NF-κB is constitutively active in canine leukemias and whether such cells are sensitive to DMAPT. Constitutive NF-κB activity (as measured by EMSA) was evident in 7 out of 8 specimens tested (supplemental figure 2A). Further, in vitro exposure to DMAPT for 24 hours resulted in decreased NF-κB activity (supplemental figure 2C) as well as decreased cell survival (36% mean viability at 10µM, n=6)(supplemental figure 2B).

After establishing that DMAPT had similar effects in spontaneous canine leukemia cells as in human cells in vitro, we proceeded to test the in vivo biological activity of DMAPT
by treating dogs with spontaneous leukemias. Notably, canine leukemias are usually only detected at advanced stages, where dogs diagnosed with acute disease have a median time to death of approximately 16 days. Thus, the clinical window of opportunity to evaluate treatment regimens is generally very brief. Nonetheless, canine studies provide a useful large animal model in which to assess basic parameters of drug activity.

DMAPT was tested in three dogs diagnosed with CD34-positive spontaneous leukemia. Both oral and intravenous routes of administration were employed over daily dosing regimens ranging from 3-12 days. A summary of the peripheral blood analysis from each study is shown in Figure 4. Although the animals were from different breeds and under different stages of supportive care (see materials and methods), we observed a rapid and consistent reduction in the level of CD34+ cells. Further, marrow analysis of one animal before and after treatment also showed a strong reduction in CD34+ cells (Figure 5A), indicating that the effects of DMAPT treatment were similar for marrow-resident vs. peripheral leukemia cells. In contrast, overall white blood cell counts were variable during the treatment regimen for each dog, with no clear reduction except in animal #2 (Figure 4B). The marked loss of CD34+ cells for each animal led us to speculate that either differentiation and/or selective ablation of more primitive cells may have occurred. Analysis of blood cell morphology showed increased levels of peripheral neutrophils, as well as a change in the morphology of blast cells (Figure 5C), supporting the concept that differentiation of the tumor was increased. Further, to assess LSC-specific effects we performed functional assays using the NOD/SCID xenotransplantation system. Analysis of pretreatment peripheral blood cells from each dog showed that leukemia specimens from animals #2 and #3 readily engrafted NOD/SCID mice (supplemental figure 3). Canine cells in NOD/SCID marrow were almost entirely CD34+ and had a blast-like morphology as determined by H&E stain, thereby indicating the leukemic origin of engrafting cells. For animal #3 we had sufficient material to compare pre and post-treatment specimens to determine whether NOD/SCID engraftment (i.e LSC activity) varied. As shown in Figure 5C (left panel), a significant reduction in the level of canine cells in NOD/SCID marrow was observed.
after DMAPT treatment (p=0.043). To further assess the self-renewal potential of canine LSC, marrow from primary NOD/SCID animals was retransplanted in secondary recipient mice (Figure 5C, right panel). These studies showed an even more profound reduction in engraftment potential for canine cells treated with DMAPT (p=0.002), suggesting that the canine LSCs were significantly impaired by the drug in vivo.

Finally, to examine in vivo biomarkers of DMAPT activity, the levels of γH2AX and the NF-κB p65 subunit were analyzed in cells from specimens before and after treatment. As shown in Figure 5D, increased γH2AX and decreased nuclear NF-κB p65 were detected post-treatment, indicating that DMAPT induced oxidative stress and inhibited NF-κB in vivo.

Discussion

In vivo targeting of LSCs represents a formidable challenge to the leukemia research field. Not only must future therapies more effectively eradicate LSCs, they must do so with less collateral damage to normal tissues. Several biological features of normal stem cells are retained in malignant populations and likely contribute to the difficulty in targeting the LSC population. For example, a mostly quiescent cell cycle status, expression of xenobiotic efflux pumps, and a protective microenvironmental niche are all factors that may shield LSC from therapeutic insult. Thus, these parameters and possibly other aspects of in vivo biology must be considered for the development of improved regimens.

From basic studies of primary human tissue we have previously proposed that two types of events are necessary to induce preferential induction of cell death in LSCs: (i) inhibition of survival signals (such as NF-κB) and (ii) activation of stress responses. Importantly, neither of these events alone appears to mediate substantial killing of LSC. We hypothesize that stress responses such as increased activity of heat shock proteins, DNA damage pathways, and oxidative stress response factors (i.e. HO-1 and Nrf2), are a direct result of drug-induced cellular damage and that increased NF-κB is a
protective reaction to the insult. Thus, by inhibiting elements of the NF-κB pathway (or similar survival factors) the detrimental effects of stress, such as increased oxidative load, are uncovered by regimens that mediate both effects. We further hypothesize the PTL-based drugs fall into this class of drug, in which both induction of stress and inhibition of survival signals are central to the therapeutic mechanism.

In the present report, we have enhanced the anti-leukemia features of PTL, by creating a more pharmacologically useful form of the drug, DMAPT; and demonstrating that the analog retains key properties of the parent molecule. *In vitro* treatment of primary human AML, ALL and CML cells with DMAPT demonstrated potent eradication of leukemic stem and progenitor cells, as well as the overall blast population (Figure 1). Importantly, functional assays demonstrated that DMAPT specifically ablated primitive human leukemia cells without impairing their normal counterparts. Together, these data indicate DMAPT is a novel therapeutic candidate for targeting human LSCs, and may have utility against a broad range of hematologic cancers.

The findings above provide a strong rationale for taking DMAPT forward to human clinical trials for leukemia. However, we were cognizant that further characterization of the drug in some type of preclinical *in vivo* model could also be of value. While numerous studies have employed human-mouse xenografts to study biological properties of stem cells in vivo, there has been almost no use of such systems for therapeutic modeling. Indeed, the metabolic differences between mouse and human physiology make pharmacological comparisons difficult. Thus, our human-mouse xenograft experiments were limited to relatively simple single-dose pharmacodynamic studies. In order to derive data more pertinent to human disease, we extended our in vivo studies to include analysis of spontaneous acute leukemia in dogs. While the analysis of canine disease is attractive because it provides a unique means to investigate authentic leukemia in a large animal system, it is also challenging due to the advanced stage in which disease is typically detected. Nonetheless, we were able to conduct three case studies, roughly equivalent to human phase I clinical trials, in which pilot feasibility and pharmacodynamic analyses were performed (Figures 4-5).
findings from those studies indicate clear in vivo activity of DMAPT, as assessed by two independent biomarkers, in good agreement with previous data from in vitro studies and the mouse xenograft model. In addition, we also observed biological changes in tumor cells suggestive of drug efficacy. In all treated animals, a rapid decrease in the percentage of CD34+ cells was detected, accompanied by an increase in the frequency of differentiated cells. More importantly, functional analysis of specimens taken from animal #3 before and after DMAPT treatment showed a clear reduction in the engraftment potential of canine LSC in the NOD/SCID xenograft system. To our knowledge, these data are the first to indicate in vivo targeting of LSC in any kind of large animal. While the underlying mechanism requires further analysis, the overall findings indicate that drug-induced differentiation may be responsible. Notably, in vitro studies using U937 myelomonocytic cells indicate sub-toxic concentrations of DMAPT may induce differentiation (Supplemental Figure 4). Further, a recent report by Gopal et al has demonstrated that parthenolide is a potent inhibitor of HDAC124, which validates the concept that parthenolide-based drugs may be epigenetic modifiers and thereby function via mechanisms that include differentiation induction.

Taken together, the data indicate that DMAPT mediates in vivo biological changes in leukemia cells that will lead to their impairment and/or death. Moreover, given the strong efficacy of the drug for AML stem and progenitor cells in vitro, we propose that a similar effect is possible in vivo, and provide preliminary evidence that LSC-specific targeting can occur in spontaneous canine leukemia. Based on these preclinical findings, its oral bioavailability, and a favorable toxicology profile, DMAPT is proceeding to human phase I clinical trials in the near future.
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Conflict of Interest Disclosure: William Matthews is President of Leuchemix, Inc. which has a financial interest in DMAPT. Christopher J. Sweeney is a stockholder in Leuchemix, Inc. Peter A. Crooks is a stockholder in Leuchemix, Inc. Craig T. Jordan is a stockholder in Leuchemix, Inc.
References

Figure Legends.

Figure 1. DMAPT induces death of primary human AML, ALL and CML cells but not normal hematopoietic cells. (A) Percent viability of primary human AML cells exposed to either 7.5µM PTL or DMAPT. Viability was measured by labeling with Annexin V and 7-AAD. Analysis of total AML vs. selected CD34+ cells are indicated. (B) Percent viability of CD34+CD38- AML cells at the indicated concentrations of DMAPT. (C) Percent viability of CD34+CD10- ALL cells at the indicated concentrations of DMAPT. (D) Percent viability on CD34+CD38- CML cells at the indicated concentrations of DMAPT. (E) Percent viability of normal CD34+ or CD34+CD38- hematopoietic cells obtained from healthy donors (CB or BM) and treated at the indicated concentrations of DMAPT. In all panels the horizontal bars represent the mean and each circle or diamond represents one specimen. Analysis of each specimen was performed in triplicate and the average was used to represent the results for a single specimen. All viability values are relative to untreated controls.

Figure 2. Progenitor/stem cell functional assays for DMAPT treated cells. (A) AML versus normal cells were treated with 5 µM DMAPT for 18 hours in suspension culture, followed by plating in methylcellulose culture. Horizontal bar represents the mean. ** p<0.001 AML vs. erythroid and AML vs. myeloid. The percent of colony-forming units (CFU) was normalized to untreated control. All assays were performed in triplicate. (B) Representative examples of the percent engraftment achieved in NOD/SCID mice receiving AML (left panel) or normal CB (right panel) cells after 18 hours of culture with or without 7.5 µM DMAPT. Each symbol represents a single animal analyzed at 6 to 8 weeks after transplantation. Mean engraftment is indicated by the horizontal bar. *** p<0.0001 DMAPT vs. untreated (UNT).

Figure 3. DMAPT induces stress responses and inhibits NF-κB. (A) Primary AML cells labeled with thiol-reactive dye mBBBr before (shaded histograms) and after (open histograms) exposure to DMAPT. (B) Confocal micrograph of primary human AML cells with treated with 7.5µM DMAPT for 2 hours. HO-1 (green) and nucleus (ToPro3,
represented in red). (C) Immunoblots (top two panels) for phospho p53ser15 (top) or actin (middle) of CD34+ primary human AML cells treated with 7.5µM DMAPT for the indicated times. Bottom panel shows an EMSA for NF-κB binding for the same treatment. (D and E) NOD/SCID mice engrafted with human AML cells 6 weeks prior to the experiment were treated with a single i.p. dose of 100mg/kg DMAPT or saline control. One hour later animals were sacrificed and BM was harvested and analyzed by confocal microscopy. Panel D shows NF-κB (p65 subunit in yellow) and panel E shows Nrf-2 (green). The nucleus is shown in blue for both panels.

**Figure 4. DMAPT decreases CD34 surface expression in canine studies.** The percentage of CD34 positive cells was determined for PB samples obtained at the indicated day of treatment (left panels, open bars). WBC counts for the same samples are shown in the right panels. The dose of DMAPT, and day and route of administration is shown on the horizontal axis for each study. (A) Canine study case #1, (B) case #2 and (C) case #3.

**Figure 5. DMAPT demonstrated biological activity in vivo.** (A) Overlays for CD34 expression in BM (top) and PB (bottom) from pre-treatment (filled histograms) and post-treatment (open histograms) specimens from case #1. (B) Percent of canine cell engraftment achieved in NOD/SCID mice receiving pre-treatment (Day 0) vs. post-treatment (Day 12) cells from case #3. Each symbol represents a single animal analyzed at 6 to 8 weeks after transplantation. Primary and secondary transplants are shown in the left and right graphs respectively. Mean engraftment is indicated by the horizontal bar. * p=0.043, ** p=0.002. (C) Blood smears for PB samples obtained at the indicated days and cases. (D) Confocal micrograph for canine cells obtained before treatment (day 0) or from day 5 after initial treatment with 50mg/kg oral dose DMAPT. Top panels show cells stained for NF-κB p65 (yellow) and ToPro3 (blue). Bottom panels show γH2AX (green) and ToPro3 (red).
A. Primary AML specimens

B. Control vs. DMAPT

C. Exposure Time

D. Saline vs. DMAPT

E. Saline vs. DMAPT
An orally bioavailable parthenolide analog selectively eradicates acute myelogenous leukemia stem and progenitor cells

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