Rational Design of a Parthenolide-based Drug Regimen that Selectively Eradicates Human Acute Myelogenous Leukemia Stem Cells

Shanshan Pei, Mohammad Minhajuddin, Angelo D’Alessandro, Travis Nemkov, Brett M. Stevens, Biniam Adane, Nabilah Khan, Fred K. Hagen, Vinod K. Yadav, Subhajyoti De, John M. Ashton, Kirk C. Hansen, Jonathan A. Gutman, Daniel A. Pollyea, Peter A. Crooks, Clayton Smith and Craig T. Jordan*

1 Division of Hematology, University of Colorado Denver, Aurora, CO, US.
2 Department of Biochemistry and Molecular Genetics, University of Colorado Denver, Aurora, CO, US.
3 Department of Biochemistry and Biophysics, University of Rochester, Rochester, NY, US.
4 Department of Medicine, University of Colorado School of Medicine, Aurora, CO, US.
5 Department of Microbiology and Immunology, University of Rochester, Rochester, NY, US.
6 Department of Pharmaceutical Sciences, University of Arkansas, Little Rock, AK, US.

Running title: Rational drug regimen to eradicate AML stem cells

To whom correspondence should be addressed: Craig T. Jordan, University of Colorado-Anschutz Medical Campus, 12700 E 19th Ave, Room 10016, Aurora, Colorado 80045, Telephone: (303) 724-8165; E-mail: craig.jordan@ucdenver.edu

Keywords: drug combination, anticancer drug, drug development, drug resistance, oxidative stress, endoplasmic reticulum stress (ER stress), parthenolide, 2-deoxy-glucose, temsirolimus, leukemia

ABSTRACT

While multi-drug approaches to cancer therapy are common, few strategies are based on rigorous scientific principles. Rather, drug combinations are largely dictated by empirical or clinical parameters. In the present study we developed a strategy for rational design of a regimen that selectively targets human acute myelogenous leukemia (AML) stem cells. As a starting point, we used parthenolide (PTL), an agent shown to target critical mechanisms of redox balance in primary AML cells. Next, using proteomic, genomic, and metabolomic methods, we determined that treatment with PTL leads to induction of compensatory mechanisms that include up-regulated NADPH production via the pentose phosphate pathway as well as activation of the Nrf2-mediated oxidative stress response pathway. Using this knowledge we identified 2-deoxy-glucose and temsirolimus as agents that can be added to a parthenolide regimen as a means to inhibit such compensatory events and thereby further enhance eradication of AML cells. We demonstrate that the parthenolide, 2-deoxy-glucose, temsirolimus (termed PDT) regimen is a potent means of targeting AML stem cells, but has little to no effect on normal stem cells. Taken together our findings illustrate a comprehensive approach to designing combination anticancer drug regimens.

INTRODUCTION

Numerous studies have documented the biological complexity of human tumor cell populations, in which genetic, epigenetic, biochemical, and metabolic properties can often be quite heterogeneous (1,2). As a result, complicated multi-drug regimens are often employed to target various components of tumor biology and/or acquired drug resistance (3,4). However, the rationale behind the design of multi-drug regimens...
is usually inconsistent and often driven by pragmatism more than scientific rigor. Thus, establishing more effective means by which to identify optimal drug regimens is an important priority, particularly with the recent emergence of a broad range of targeted agents.

To enhance the design of combination regimens, we sought to draw upon advanced methods of global cell analysis that allow comprehensive studies of genomic, proteomic, and metabolomic properties. Such strategies have become prevalent in recent years and are now routinely used in nearly all types of biological research. However, to our knowledge, simultaneous use and integration of multiple “omic” methods to design drug combinations for cancer have not been widely reported. Thus, in the present study our goal was to develop a multifaceted approach that would utilize several platforms for comprehensive cell analysis.

As a model system in which to pilot this approach, we chose targeting of human acute myelogenous leukemia (AML). Previous studies have clearly established the biological heterogeneity of AML as well as sophisticated experimental systems to evaluate the efficacy of candidate regimens (5-8). Further, among human tumors, AML has arguably the best-characterized cancer stem cell population. Eradication of the AML stem cells is thought to be critical for achieving improved clinical outcomes (9-13). While several strategies have been reported (14-19), as yet, it is not clear to what extent any approach will comprehensively eradicate heterogeneous AML stem cell populations. Hence, the studies described here were designed to identify and target fundamental conserved components of AML stem cell biology.

As a foundation for rational design of a multi-drug regimen, we chose to evaluate parthenolide (PTL), a naturally occurring small molecule with reported anti-tumor properties in nearly all major forms of human cancer (20,21). Of particular interest is the activity of PTL as an agent in AML where it can target bulk tumor cells as well as the relatively rare AML stem cell population (22). These properties have made PTL an exciting pre-clinical compound to study. However, the use of PTL as a single agent to treat cancer still has some potential drawbacks. First, data from the Cancer Therapeutics Response Portal (http://www.broadinstitute.org/ctrp/) show that certain tumor types still display relative resistance to PTL treatment (23). Second, PTL has relatively unfavorable aqueous solubility and stability (18,24), thus there is a strong rationale for strategies to enhance the anti-cancer properties of PTL at as low a dose as possible. Third, given the complexity of most human tumors, it is likely that combinatorial strategies using drugs to target multiple intracellular pathways will yield superior results. Thus, PTL makes an excellent starting point to test our strategy for the rational design of a multi-drug regimen.

The first step of our strategy involved comprehensive characterization of the anti-leukemia mechanism of PTL in AML. Although previous studies by our group and others have shown that PTL is a strong inhibitor of the NF-κB pathway and also a potent inhibitor of glutathione metabolism (22,25-29), there have been very few studies that systematically characterize its anti-cancer mechanism at a multi-omic level. In the present study, we investigated the mechanism of PTL by first using a biotinylated analog to identify its proteomic interactome. In addition, we profiled PTL-induced transcriptomic changes in primary AML cells. Further, we employed isotope labeling experiments to monitor global metabolic flux changes that occur upon treatment of primary AML cells with PTL. By integrating results from these omic studies along with our previous reports, we discovered that PTL directly modulates several components of AML redox balance, and induces strong activation of the Nrf2-mediated oxidative stress response as well as up-regulation of the pentose phosphate pathway (PPP), both of which are thought to be compensatory mechanisms that are employed by AML cells in an effort to survive the PTL insult. Therefore, we tested the concept that inhibition of such compensatory events would enhance the cytotoxicity of PTL. Subsequent studies identified the triple drug regimen “PDT” in which parthenolide (P) was combined with 2-deoxy-glucose (D), and temsirolimus (T), drugs chosen for their ability to inhibit the PPP and the Nrf-2 mediated anti-oxidant response, respectively (30,31). Biological analyses of the PDT regimen
demonstrated strong toxicity towards primary AML cells including those that are relatively resistant to PTL treatment alone. Importantly, the PDT regimen also displays potent toxicity towards leukemic stem/progenitor cells with very little toxicity towards normal hematopoietic counterparts. Thus, by using a targeted omic approach, we were able to perform rational design of the PDT regimen with a superior leukemia-specific cytotoxicity compared to its parent compound, parthenolide.

RESULTS

Identification of the proteomic interactome of PTL in primary AML cells — As an initial step towards developing enhanced PTL-based combination regimens, we first sought to thoroughly characterize the proteomic interactome of PTL. The most prevalent chemical feature of PTL is an alpha-methylene-gamma-lactone moiety, which mediates covalent interactions via Michael addition reaction (Figure 1A). Hence, PTL’s cellular targets should be readily identifiable using biochemical pull-down methods. To this end, we have previously employed a biotinylated analog of PTL, known as MMB-biotin (Figure 1B), to identify candidate target proteins. We have validated its use for known targets of PTL including IKKβ and NF-κB p65 (26-28). In the current study, we combined the MMB-biotin-based pull-down assay with liquid chromatography-tandem mass spectrometry (LC-MS/MS) to allow the identification of the entire proteomic interactome of PTL in primary AML cells.

Briefly, we treated primary AML cells in culture with the MMB-biotin probe or free biotin control for 2 hours, followed by washing and harvesting steps. We then fractionated MMB-biotin/free biotin-treated cells into cytosolic, membrane, nuclear, and insoluble cytoskeletal fractions to allow identification of PTL-binding targets from each distinct subcellular compartment (Figure 1C). Purity of each fraction was confirmed by showing enrichment of fraction-specific markers (Figure 1D). Each fraction was then incubated with streptavidin-coated beads to isolate PTL-binding targets. We confirmed the specificity of our pull-down by probing each pull-down product with streptavidin-HRP (SA-HRP). As shown in Figure 1E, lane 1, there are only two bands detected by SA-HRP from pull-down products of free biotin treated cells: one at around 75KD which is a previously described non-specific binding event (labeled as n.s.) (26), another at around 12KD which is presumably a monomeric SA dissociated from SA-coated beads (labeled as SA). In contrast, pull-down products from MMB-biotin treated cells have a spectrum of binding targets between 20-100KD (Figure 1E, lane 2). In addition, these targets are largely distributed in the cytosolic and membrane fractions, with much less in the cytoskeletal fraction, and the least in the nuclear fraction (Figure 1E, lane 3-6). Using LC-MS/MS of each fraction, we subsequently identified 312 binding targets of PTL in primary AML cells (Supplemental Table 1). Notably, several previously reported binding targets of PTL including IKKβ, GCLC, GPX1, and TXN were all successfully identified through this approach (25-27), suggesting our method had good specificity and coverage. Subsequently, we employed the Ingenuity Pathway Analysis (IPA) software and identified a list of pathways/biological functions that are significantly represented by this pool of binding targets (Supplemental Table 2). Importantly, the Nrf2-mediated oxidative stress response (p<0.0005) and glutathione metabolism (p<0.005) pathways are highly enriched, consistent with our previous findings that the ability of PTL to target glutathione metabolism and induce oxidative stress is important for its anti-leukemia activity (22,25).

Characterization of the transcriptomic changes induced by PTL in AML cells — We next wanted to characterize PTL-induced transcriptomic changes in primary AML cells. To this end, we treated primary AML cells (n=4) with 7.5 μM PTL or DMSO control for 6 hours, isolated total mRNA, and profiled the expression of their transcriptomes using RNA-seq. By comparing the gene expression level between DMSO control and 7.5 μM PTL treated cells, we identified a total of 2114 significantly dysregulated genes (Fold change ≥ 1.5, p<0.05, Supplemental Table 3). We then used the IPA software to predict the significantly dysregulated pathways in PTL-
Rational drug regimen to eradicate AML stem cells

Among the top 5 most dysregulated pathways are the Nrf2-mediated oxidative stress response pathway (hereafter referred to as the Nrf2 pathway) and the protein ubiquitination pathway (hereafter referred to as the Ub pathway). This finding is consistent with our previous study using a different cohort of primary AML specimens (GEO deposit ID: GSE7538) (32,33), suggesting these anti-leukemic activities of PTL are universally present irrespective of heterogeneous primary AML specimens.

To test if there is a link between the PTL proteomic interactome and PTL-induced transcriptomic changes, we examined the pool of 312 PTL-binding targets for ones that are directly present in the top 5 transcriptionally dysregulated pathways induced by PTL (Figure 1F). This analysis identified 13 (p<0.0005) PTL-binding targets in the Nrf2 and 14 (p<0.0005) targets in the ubiquitin pathway (Figure 1G), suggesting that proteomic interactions of PTL may be linked to PTL-induced transcriptomic changes. Importantly, the up-regulation of the Nrf2 pathway suggests an accumulation of cellular oxidative stress (34), while the activation of the Ub pathway usually implies an increase of protein misfolding stress (35). Thus these data essentially suggest two strong anti-leukemic activities of PTL including the induction of cellular oxidative and protein misfolding stress.

Metabolomic analyses reveal increased PPP activity for NADPH production in PTL-treated AML cells — To further investigate the anti-leukemic activity of PTL, we characterized PTL-induced metabolomic changes in primary AML cells using previously established methods (36). As outlined in Figure 2A, primary AML cells were cultured in media containing uniformly labeled U-13C6-glucose or dual-labeled 13C1,2-glucose, and treated with vehicle control or 7.5 μM PTL. We harvested cells at 0, 4 and 6 hours and quantified the relative distribution of the isotopologue of 13C-labeled metabolites at each time point.

As shown in Figure 2B, compared to vehicle control, 6 hour PTL-treated AML cells have increased levels of several important metabolic intermediates including 13C-glucose-6-phosphates (M+6, 5.36 fold), 13C-ribose-phosphate (M+5, 2.80 fold), 13C-glyceraldehyde-3-phosphate (M+3, 15.04 fold), and 13C-lactate (M+3, 1.83 fold), suggesting primary AML cells respond to PTL insult by increasing flux of glucose through glycolysis and the pentose phosphate pathways (PPP). In addition, we also observed increased levels of citric acid (TCA) cycle intermediates including 13C-citrate (M+2, 4.67 fold), 13C-malate (M+2, 4.23 fold), and 13C-asparate (M+2, 1.47 fold), further suggesting that PTL-treated AML cells become more glucose addicted than control-vehicle treated cells, as they appear to rely on glucose oxidation to fuel energy-generating pathways.

The increase of glucose flux into the PPP is particularly interesting, since the PPP is a major pathway generating NADPH, which is important for glutathione homeostasis (37). We have previously reported that 4-6 hour PTL (7.5 μM) treatment can induce maximum glutathione depletion in primary AML cells (25). Thus we hypothesized that the observed increase of glucose flux into the PPP at around 6 hours is used to generate NADPH to rescue PTL-induced glutathione depletion. To test this hypothesis, we first confirmed the PPP activity increase in PTL-treated AML cells by a second set of labeling experiments using the dual-labeled 13C1,2-glucose. In this particular experiment, since one carbon atom is lost in the form of CO2 during glucose oxidation steps through the PPP (38), the M+1 isotopologue of 13C-glyceraldehyde-phosphate is exclusively generated by glucose flux through the PPP, not glycolysis. Thus, the level of the M+1 isotopologue represents the PPP activity. As shown in Figure 2C (complete data set in Supplemental Figure 1), this M+1 isotopologue of 13C-glyceraldehyde-phosphate is dramatically increased over time in PTL-treated AML cells, while its level in vehicle control-treated cells remains virtually zero (p<0.001). Next we tested the hypothesis that the increased PPP activity is used to produce more NADPH. To this end, we performed a separate bioluminescent assay to quantify the cellular NADPH and NADP+ levels and found that 7.5 μM PTL treatment indeed significantly elevated both net NADPH level and NADPH/NADP+ ratio in AML cells (Figure 2D). Together these data strongly demonstrate that
AML cells respond to PTL insult with a robust activation of the PPP for NADPH production presumably to detoxify PTL-induced oxidative stress in primary AML cells.

Integration of ‘omics’ data provides strategies for rational design of PTL-based anti-leukemic regimens — The metabolomic data above reveals that activating glucose metabolism to shunt more glucose into the PPP for NADPH production is a major metabolic response of AML to PTL insult. This observation is consistent with our transcriptomic study results, which show that PTL dramatically induces Nrf2-mediated oxidative stress response (Figure 1G). Thus both metabolomic and transcriptomic studies shed light on the robust oxidative stress induced by parthenolide and some of the prominent responses mounted by primary AML cells upon challenge with PTL. In addition, our proteomic study also shows that PTL can directly interfere with many proteins that are present in the Nrf2 pathway (Figure 1G), and our previous study has reported that PTL can potently deplete the cellular antioxidant glutathione (25), thereby providing two additional mechanistic insights to explain PTL-induced oxidative stress. Thus through the integration of multiple omic techniques performed in the current study along with previous research, we hypothesize that among the many anti-leukemic activities of PTL, its ability to induce oxidative stress is the most important. Notably, oxidative stress is known to induce protein-misfolding stress since correct folding of proteins is usually redox-sensitive (39,40). Therefore, we also hypothesize that the increase of protein misfolding stress upon PTL treatment is a secondary consequence of PTL-induced oxidative stress.

Importantly, the above analyses of the anti-leukemic activities of PTL provide us with strategies for rational design of PTL-based anti-leukemic regimens. As illustrated in Figure 3A, we reasoned that addition of drugs that can enhance PTL-induced oxidative stress and possibly protein misfolding stress represent a promising strategy to further increase the anti-leukemic activity of PTL. To this end, we tested 2-deoxy-glucose (2DG), a hexokinase inhibitor that can block glucose oxidation via the PPP pathway responsible for NADPH production (30), and Temsirolimus (TEM) that can effectively abrogate PTL-induced Nrf2-mediated oxidative stress responses (31). We hypothesized that the combined use of PTL + 2DG + TEM (termed the PDT regimen) would achieve superior eradication of primary AML cells due to simultaneous induction of oxidative stress and inhibition of the compensatory anti-oxidant responses intrinsic to AML.

The PDT regimen blocks both Nrf2 response and NADPH production thereby increasing the potency of PTL — To initially evaluate activity of the PDT triple drug regimen in AML cells, we investigated the impact of PDT on several aspects of the AML redox system, including glutathione, oxidative stress, Nrf2 pathway response, and NADPH. Importantly, for these experiments and all subsequent analyses in the current study, we employed a lower dose of PTL (2.5 μM), which is not sufficient to induce significant anti-leukemic activity alone. Using a lower dose permits better detection of additive/synergistic interactions that result in enhanced PTL activity. Further, even though 2.5 μM PTL was used for the drug combinations, the single agent PTL control for these studies was maintained at 7.5 μM, which is generally a potent cytotoxic dose for AML cells (22,25).

As shown in Figure 3B, while each drug alone had a minimal effect in depleting cellular glutathione, they synergized and depleted >80% of total glutathione in primary AML cells. This degree of glutathione suppression is similar to what we have previously observed using PTL alone at 7.5 μM (25). Thus, the addition of 2DG and TEM restores ability of a low dose PTL to target glutathione metabolism, a central mechanism in the overall AML-specific toxicity of PTL-based anti-leukemic strategies. Further, we note that a biological consequence of the glutathione inhibition obtained in PDT-treated AML cells is a strong increase in production of reactive oxygen species (ROS), shown by CM-H₂DCFDA staining (Figure 3C). Comparing to 7.5μM of PTL, the PDT treatment induced significantly stronger ROS increase at all time points. And interestingly, unlike a gradually
decreased ROS profile in 7.5uM PTL-treated AML cells, the ROS increase in PDT-treated AML cells was sustained up to 8 hours post PDT treatment, suggesting a lack of Nrf2-mediated oxidative stress response, therefore disabling AML cells from managing the elevated ROS. To test this hypothesis, we treated 4 primary AML specimens with the PDT combination for 6 hours and performed QPCR analysis on genes in the Nrf2 pathway. For comparison purpose, we also profiled 6 hours, 7.5 uM PTL-induced gene expression changes in these cells side-by-side. As shown in Figure 3D, our data demonstrate that 7.5 uM PTL induced strong up-regulation of all glutathione pathway components including the cysteine transporter SLC7A11/SLC3A2 complex, the rate-limiting glutathione synthesis enzyme complex GCLC/GCLM, and glutathione reductase GSR. This is consistent with our previous finding that 7.5 uM PTL can potently deplete glutathione in AML cells, and indicates that up-regulation of these genes is a component of AML response to PTL (25). In contrast, despite the strong glutathione depletion shown in Figure 3B, PDT demonstrated almost no induction of GCLC/GCLM and GSR. We propose that the lack of this compensatory response to drug insult may explain the sustained ROS increase seen in PDT-treated AML cells (Figure 3C). In addition to the glutathione system, we also investigated the expression of HMOX1, another major effector of the Nrf2-mediated anti-oxidant response. As shown in Figure 3D, while HMOX1 mRNA was strongly up-regulated by 7.5 uM PTL, it is only mildly induced by PDT. Consistent with the mRNA expression data, PTL treatment produced a dramatic increase in HMOX1 protein expression, while the PDT regimen only induced a modest increase (Figure 5E). Finally, we observed that 7.5 uM PTL induced the expression of thioredoxin system proteins: TXN and TXNRD1, and suppressed the expression of thioredoxin inhibitory protein TXNIP in AML. However, PDT did not alter the expression of these thioredoxin system genes as well (Figure 3D).

We next looked at Nrf2 downstream genes that are specifically related to NADPH production. Shown in Figure 3F, while 7.5 uM PTL induced strong up-regulation of the PPP enzymes G6PD and PGD, PDT treatment did not induce them. Consequently, while 7.5 uM PTL induced a dramatic NADPH increase in AML cells, PDT did not elevate cellular NADPH level during 8 hours of its treatment (Figure 3G), suggesting a lack of NADPH production from PDT-treated AML cells despite a >80% loss of cellular glutathione (Figure 3B).

Taken together, these data indicate that the PDT regimen is a potent ROS inducer in primary AML cells, but despite the rapid increase in oxidative stress, PDT-treated cells are unable to mount an effective anti-oxidant (i.e. protective) response. These findings predict that cytotoxicity towards AML cells should be increased for the PDT regimen in comparison to PTL.

**PDT regimen does not affect NF-κB signaling but induces strong protein misfolding stress in primary AML cells** — PTL is also known as a strong NF-κB inhibitor (20). However, although we confirmed that 7.5 uM PTL induced loss of p65 EMSA binding activity, reduction of NF-κB p65 phosphorylation, and decrease in NF-κB target gene IL-6 expression, the PDT regimen had no effect on any of these three readouts, indicating that the anti-leukemic mechanism of PDT is independent of NF-κB inhibition (Figure 4A, B, C). It further suggests that the anti-leukemic mechanisms of PDT are similar but not identical to PTL alone. Given these differences, we sought to develop a more global knowledge of the overall PDT mechanism, and therefore conducted a transcriptome analysis. For these studies we profiled 6-hour PDT-induced gene expression changes in four primary AML specimens using RNA-seq and then performed bioinformatic analysis to characterize key signaling pathways that are related to the anti-leukemic activities of PDT.

Using a collection of KEGG gene sets, we performed Gene Set Enrichment Analysis (GSEA) of our RNA-seq data and found that the “Proteasome” function was strongly up-regulated by the PDT treatment (Figure 4D, left panel, Supplemental Table 5). Since increase of proteasome activity usually is a cyto-protective response of cells to degrade misfolded proteins...
Rational drug regimen to eradicate AML stem cells

(41), this data suggests that the PDT treatment induces protein misfolding stress in primary AML cells. Consistent with this observation, in an independent GSEA analysis using a collection of REACTOME gene sets, we also found that the “Unfolded protein response” pathway is significantly up-regulated upon PDT treatment (Figure 4D, right panel). And lastly, we also analyzed the RNAseq data with the iPAGE gene expression analysis tool (42). This analysis identified the “unfolded protein binding” function as being significantly up-regulated in 3 out of 4 primary AML specimens treated with PDT (Supplemental Figure 2A).

To confirm the results from these gene expression analyses, we next examined the expression level of individual genes related to proteasome activity, chaperone function, and ER stress response, specifically. As shown in Figure 4E, a global up-regulation of chaperone proteins (e.g. HSP60, HSP70, HSP90, and DNAJs, etc.) was seen in PDT-treated AML cells. In addition, expression of many proteasome subunits was also increased in PDT-treated AML cells. Moreover, we observed global increase of well-known ER stress markers (SERCA2, PDIs, GRP94, GRP78, and CHOP) upon PDT treatment. Lastly, CHOP up-regulation is typically considered to be a marker for ER stress that readily triggers apoptosis (43). Therefore, we performed more detailed quantitative PCR analysis on the expression of CHOP following PDT treatment. As shown in Figure 4F, while each drug alone has a small effect on CHOP expression (1.7-3.6 fold), together they induced an impressive 12.7-fold increase in CHOP gene expression, suggesting a synergistic ER-stress induction effect by PDT. Together these analyses suggest that the PDT regimen creates a strong protein misfolding stress in primary AML cells.

In addition to up-regulated pathways, our GSEA analysis also identified biological functions that are significantly down-regulated by PDT (Supplemental Table 6). Among them are results suggesting down-regulation of DNA replication and fatty acid metabolism (Supplemental Figure 2B,C). DNA replication is important for proliferation of cancer cells; thus PDT-induced inhibition of DNA replication is potentially important to its activity towards bulk leukemic cells. On the other hand, fatty acid metabolism is known to be important for leukemia stem cells (44). Thus, inhibition of fatty acid metabolism induced by PDT could contribute its ability to target AML stem/progenitor cells.

The triple drug regimen PDT can effectively target AML stem and progenitor populations — Given that the PDT triple drug regimen has a unique ability to induce ROS and ER stress, and simultaneously inhibits many cytoprotective antioxidant responses of AML, we hypothesized that the PDT combination would demonstrate strong anti-leukemic activity. To test this hypothesis, we treated 10 primary AML specimens with single, dual, and triple drug combinations for 24 hours and measured the viability of cells after each treatment. As presented in Figure 5A, our data showed that the viability of primary AML cells was modestly affected when treated with each drug alone at a sub-lethal dose: 2.5 µM PTL (87.7±13.8% viable), 0.1125 mg/ml 2DG (67.5±19.5% viable), and 2.5 µg/ml TEM (85.6±13.2% viable). Dual drug combinations yielded somewhat more efficient targeting: PTL+2DG (45.3±23.0% viable), PTL+TEM (63.3±16.1% viable), and 2DG+TEM (37.1±14.7% viable). However, treatment with triple drug regimen PDT showed the most effective eradication of AML cells (19.5±10.2% viable), demonstrating that the combination of all three drugs is substantially more toxic to primary AML cells than single and dual drug treatments (Figure 5A). Importantly, we have also used PDT to treat primary AML cells that are especially resistant to single agent PTL. As shown in Figure 5B, while 7.5 µM PTL treatment only resulted in about 25% median cytotoxicity, PDT was much more effective, inducing approximately 75% cell death, demonstrating that the PDT triple drug regimen can be superior to a single agent PTL.

While targeting of bulk leukemia cells is certainly desirable, for AML the most critical targets are the more primitive stem and progenitor cell populations, which are thought to propagate the disease (10-13). To directly determine if the PDT combination can functionally impair progenitor populations, we treated primary AML cells with various combinations of PTL, 2DG, and
TEM drugs for 24 hours in culture and then plated them for colony-formation assays (to detect progenitor cell activity). Our results showed that PDT treatment greatly reduced the colony-forming potential of primary AML cells, and was consistently superior to a 7.5 μM PTL alone (Figure 5C). Together, these data clearly indicate that, in addition to its toxicity to bulk leukemia cells, the PDT regimen can also strongly impair the more primitive progenitor cell population of primary AML.

The PDT regimen spares normal hematopoietic stem and progenitor cells — PTL was originally characterized as an anti-AML agent based in part on its ability to selectively target malignant but not normal hematopoietic cells (22). Thus, it was important to determine the relative activity of the PDT regimen towards normal cells as well. To this end, we isolated mononuclear cells (MNCs) from normal bone marrow donor specimens and performed experiments analogous to the AML studies described in Figure 5. Our results showed that all single, dual, and even the triple drug regimen PDT (94.3% viable) had only minimal toxicity towards total MNCs (Figure 6A,B), indicating no overt toxicity to bulk normal bone marrow cells. More importantly, the CD34+ population, which contains normal hematopoietic stem and progenitor cells (45), also showed no significant toxicity to PDT treatment (93.8%, Figure 6B,C). To readout functional abilities, we also performed CFU assay and demonstrated no significant targeting of normal progenitor cells (Figure 6D).

The PDT regimen is selectively toxic to leukemia stem cells — Finally, to measure ability of the PDT regimen to target the leukemia stem cell population of primary AML, we treated primary AML cells with various combinations of PTL, 2DG, and TEM drugs for 24 hours ex vivo and transplanted them into immune-deficient NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice (to detect leukemia-initiating cell activity). We found that the ability of PDT-treated AML cells to engraft and repopulate in NSG mice decreased significantly (7.9±1.9%) compared to either untreated (61.7±4.2%), PTL alone (58.4±7.5%), or dual drug combinations: PTL+2DG (27.0±8.0%) and PTL+TEM (22.8±13.3%) (Figure 7A). In contrast, same treatment with the PDT triple drug regimen resulted in no effect to the engraftment ability of normal mononuclear cells, indicating a minimal toxicity of the PDT regimen to normal hematopoietic stem cells (Figure 7B). These results strongly indicate that the PDT triple drug regimen designed by our rational approach represents a potent and selective means to selectively target both bulk and stem/progenitor populations of primary AML.

DISCUSSION

The primary goal of this study was to demonstrate the rational design of a novel drug combination regimen to target human AML stem and progenitor cells. This is a challenging objective because of the well-known cellular and molecular heterogeneity within different subpopulations of primary AML cells (1,2). In particular, functionally defined AML stem cells are biologically distinct from bulk leukemic blast cells and are often less responsive to many forms of conventional therapy (10-13). Hence, an improved therapy must target broadly conserved properties among AML subpopulations, including the stem cell subsets. In addition to the heterogeneity of AML, lack of systematic methodologies to design complex yet effective drug combinations is another major challenge in the drug discovery field.

In the current study, we addressed these challenges by employing and integrating multiple methods of global cell analysis to inform rational design of effective anti-leukemic regimens. To pilot our approach, we chose parthenolide (PTL), a compound that has already been shown to be effective against AML stem cells (22), as a foundation for designing a novel drug combination with superior anti-leukemic properties. Our global cell analysis methods involve multiple omic studies designed to thoroughly characterize the anti-leukemic mechanisms of PTL. To begin, we used a biotinylated analog of PTL to provide the first comprehensive characterization of the PTL proteomic interactome. Next, we profiled PTL-induced gene expression changes in primary AML cells at the transcriptome level. By analyzing the
results from proteomic and transcriptomic studies together, we found that although the overall repertoire of PTL-binding candidates is relatively broad, there is a striking prevalence for proteins directly involved in the Nrf2-mediated stress response and the protein ubiquitination pathways, which are significantly dysregulated by PTL. These findings suggest a strong link between the proteomic interactome and the transcriptomic response of PTL in AML cells. Most importantly, these data provide an unbiased means by which to pinpoint key anti-leukemic activities of PTL, including induction of oxidative and protein misfolding stress.

We then employed a comprehensive metabolomic analysis to further characterize the anti-leukemic mechanism of PTL. Both our uniformly labeled U-13C6-glucose and dual-labeled 13C1,2-glucose experiments clearly demonstrate a strong increase in the PPP activity upon PTL treatment, presumably for the production of NADPH. Notably, NADPH is also known to be produced by other metabolic steps including the conversion of malate into pyruvate catalyzed by the NADP-dependent malic enzyme 1 (ME1) and the conversion of citrate into alpha-ketoglutarate catalyzed by the isocitrate dehydrogenase 1 enzyme (IDH1) (46). In our current study, although we cannot exclude the possibility that these processes may also contribute to NADPH generation, given the strong increase of the PPP activity shown in Figure 2B, 2C, and Supplemental Figure 1, we propose that the increased PPP activity is a major factor contributing to elevated NADPH production.

In addition to the strong increase in the PPP, our metabolic analysis also shows global increase of glycolysis, lactate production, and OXPHOS. Given that the glycolysis, PPP, and OXPHOS pathways are all interlinked by metabolic intermediates, we propose that the increase in OXPHOS and glycolysis intermediates is a likely result of the PPP feeding back to glycolysis and eventually into the TCA cycle. Importantly, the increase in the PPP is relevant because we also observed a clear NADPH increase upon PTL treatment. In addition, we showed, in our dual-labeled 13C1,2-glucose experiment, that the M+1 isotopologue of 13C-glyceraldehyde-3-phosphate (could only be generated by the PPP) is specifically elevated in PTL-treated AML cells but not in untreated cells (Figure 2C and Supplemental Figure 1). These studies demonstrate that the most dominant metabolic response of AML cells to PTL treatment is the up-regulation of the PPP for NADPH production, which is known to detoxify oxidative stress (37). Thus together our metabolomic data further supports induction of oxidative stress as a central component of PTL’s anti-leukemic activity. Consequently, we used oxidative stress as the focal point in the rational design of PTL-based drug combinations.

With a detailed understanding of the PTL mechanism in hand, we further integrated the analyses from all omic studies, and identified two key opportunities for developing an enhanced PTL-based drug regimen: 1) the Nrf2-mediated oxidative stress response pathway (readily evident from analyses of both PTL’s proteomic interactome data and PTL-induced transcriptome change data), and 2) the PPP (metabolomic data showed a dramatic increase of PPP activity as a major cytoprotective response of AML to PTL insult). We hypothesized that addition of drugs to simultaneously inhibit both the Nrf2-mediated anti-oxidant response and the PPP would enhance the selective anti-leukemic activity of PTL. To this end, we designed a triple drug regimen termed PDT, which is composed of parthenolide, 2-deoxy-glucose (to inhibit NADPH production from the PPP), and temsirolimus (previously shown to inhibit Nrf2-mediated responses in AML cells (31)). We showed that this PDT regimen displayed potent toxicity towards both bulk and stem/progenitor populations of primary AML cells, but very limited toxicity towards the normal hematopoietic cells. Notably, the PDT regimen was effective even for AML specimens that were relatively resistant to PTL alone. Thus, not only was PDT more active towards AML cells, it was also more broadly effective amongst varying AML specimens.

From a mechanistic perspective, the anti-leukemic activity of PDT is associated with its strong ability to induce oxidative stress without activating the compensatory Nrf2 and PPP responses in primary AML cells. We postulate this activity at least partly explains its selectivity,
because multiple studies have suggested that an aberrant redox homeostasis is a common characteristic of primary AML cells compared to normal hematopoietic cells. For example, we have previously shown that CD34+ primary AML cells (enriched for stem and progenitor AML cells) and CD34+ normal hematopoietic cells have substantial differences in levels of reduced glutathione and relative response to PTL insult. Intriguingly, the steady state level of reduced glutathione is higher in normal cells, and is also restored more quickly upon oxidative insult (25). Thus, it appears that AML cells exist in an intrinsically higher oxidative state and also have less capacity to manage further oxidative stress. Our findings are supported by other studies, which demonstrate that oxidative stress is a common property of AML and increased oxidative stress appears to be a key event during leukemic transformation (47-49). Thus it is likely that AML stem cells are more sensitive to PDT-induced oxidative stress due to their higher basal level of ROS relative to normal hematopoietic stem cells. Further, as shown in the present study, by disabling compensatory responses of AML (Nrf2 and PPP), the activity of agents such as PTL can be substantially enhanced.

Interestingly, addition of temsirolimus seems to inhibit parthenolide’s effect in reducing NF-κB p65 phosphorylation. We think this is likely caused by a crosstalk between oxidative stress and NF-κB signaling. Both the current study and previous studies have shown that temsirolimus can induce oxidative stress by suppressing antioxidant responses, and oxidative stress can activate NF-κB signaling (31,50). Thus we think that addition of temsirolimus rescued PTL-induced NF-κB inhibition through suppression of antioxidant response and subsequent induction of oxidative stress that finally reactivated the NF-κB signaling. As a consequence, PDT-induced cell death in primary AML cells is independent of NF-κB inhibition. Although primary AML cells are known to have constitutively active NF-κB activity that can be targeted to enhance cell death (22,51), our data suggest that it is also possible to induce severe cell death in primary AML cells by generating other forms of cytotoxic stimuli such as protein misfolding stress.

We show in the current study that in addition to generating oxidative stress, PDT can also induce strong protein misfolding stress in both the cytoplasm and ER of primary AML cells. Protein misfolding stress in the cytoplasm is usually detoxified by chaperone proteins, which refold misfolded proteins, and proteasomes which degrade and recycle misfolded proteins (52,53). On the other hand, protein misfolding can also happen in the ER where nascent protein synthesis takes place. The accumulation of misfolded proteins in the ER can trigger the “death arm” of the unfolded protein response (UPR), thereby leading to apoptosis (54). In our study, we observed global up-regulation of chaperone proteins, proteasome subunits, and ER stress markers in PDT-treated primary AML cells, indicating a strong ability of PDT to induce protein misfolding stress in AML cells. Importantly, studies have suggested that leukemia cells might already be under higher protein misfolding stress compared to normal hematopoietic cells. For example, Dai et al have reported that heat shock proteins are often up-regulated in cancer cells and are particularly high in hematopoietic malignancies (55). Our own studies have also found that the expression of HSP60, 70, and 90 were consistently higher in multiple primary AML cells compared to CD34+ normal hematopoietic cells (data not shown). Consistent with these observations, HSP90 inhibitors 17-AAG and 17-DMAG have both been shown to be effective against AML cells (56). Together, these data suggest that PDT-induced protein misfolding stress might be another mechanism underlying its potency and selectivity against primary AML specimens.

Simultaneous induction of multiple types of stress that can converge to synergistically promote apoptosis is an advantage of using drug combinations to treat cancer. From this perspective, we propose that the oxidative stress and protein misfolding stress induced by PDT are likely inter-connected and collaborate to create amplified apoptotic signaling in primary AML cells. This reasoning is largely based on the fact that proper protein folding is a redox-sensitive process subjected to redox-modifications including S-glutathionylation, carbonylation etc. (39,40).
Thus, excessive exposure of the AML proteome to sustained oxidative stress will likely lead to dramatic protein misfolding stress increase as a consequence.

Finally, we summarize the proposed mechanism of PDT-mediated cytotoxicity in the diagram shown in Figure 7C. Briefly, our data show that PTL mediates a potent inhibition of glutathione metabolism (25), which triggers activation of Nrf2-mediated oxidative stress response as well as increased utilization of the PPP for NADPH production. 2DG blocks the PPP, reduces the production of NADPH, and thus inhibits the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH). In parallel, TEM blocks translocation of Nrf2 to the nucleus thereby inhibiting the synthesis of anti-oxidant machineries (i.e. GCLM, GPX1, TXN), which leads to further ROS increase (31). Although not directly examined in the present study, it is likely that the increase in ROS promotes ER stress via a redox-mediated mechanism (39,40). And finally, PDT-induced oxidative and protein misfolding stress synergize to induce potent cell death in primary AML cells. Importantly, because AML cells are likely more susceptible than normal cells to increases of ROS and protein misfolding stress, we propose that these mechanisms also explain the selectivity of the PDT regimen.

**EXPERIMENTAL PROCEDURES**

**Human Specimens**
AML specimens were obtained from apheresis product, peripheral blood, or bone marrow of AML patients who gave informed consent for sample procurement on the University of Colorado tissue procurement protocol. Normal bone marrow (NBM) and normal cord blood (NCB) specimens were obtained from volunteer donors who gave informed consent on a research review board approved protocol at the University of Colorado. Total mononuclear cells (MNCs) were isolated from NBM or NCB donor specimens by standard Ficoll procedures (GE Healthcare). If needed, total MNCs were further enriched for CD34 positive cells using the MACS CD34 enrichment kit (Miltenyi Biotec). See supplemental table 7 for additional details on human specimens.

**Cell culture and drug treatments**
Primary human AML cells and MNCs were cultured and treated at 1 million cells per ml in IMDM (Life technologies)-based serum free media (SFM) at 37 °C, 5% CO2 incubator as previously described (25). Cells were pre-incubated in SFM media for 1 hour before treatment with parthenolide (Enzo), temsirolimus (Pfizer), and 2-deoxy-glucose (Sigma) at desired concentrations. For all drug combinations, drugs were added at the same time.

**Preparation of Subcellular fractions and total cell lysates**
Subcellular fractionation of primary AML cells was performed using the Qproteome Cell Compartment Kit (Qiagen) per manufacturer’s protocol. Total cell lysates were made by performing cell lysis in Buffer F (10 mM Tris–HCl pH 7.5, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 5 uM ZnCl2, 1% Triton X-100, freshly add 1mM PMSF, 1x PIC, and 0.1 mM Na3OV4) at 10-20 million per ml.

**Streptavidin pull-down assay**
Total cell lysates and subcellular fractions from MMB-biotin treated cells were incubated with streptavidin (SA) beads (Thermo) at 4 °C for 2 hours to pull down binding targets of PTL. After incubation, beads were sequentially washed one time with PBS, three times with high salt wash buffer (500 mM NaCl in 0.1 M pH 5.0 NaOAc), three times with low pH wash buffer (0.1 M pH 2.8 glycine–HCl), and one last time with PBS. Finally, the SA beads were boiled for 10 min in 2× SDS–PAGE sample buffer to elute all pull-down products for Western blot analysis, or digested with trypsin to allow subsequent LC-MS/MS-based identification of targets.

**LC-MS/MS based identification of the proteomic interactome of PTL**
After SA pull-down, on-bead trypsin digestion was performed to generate peptide fragments of targets. These fragments were analyzed by nanospray LC-MS/MS, using Magic C18 AQ reverse-phase liquid chromatography resin (Michrom BioResources), custom packed into 5 cm x 75 mm
fused silica column, which was coupled in-line to an ion trap mass spectrometer (Finnigan LTQ, Thermo). MS/MS-acquired data were searched against human amino acid sequences within the NCBI protein database, using the MASCOT software (Matrix Science). This method identified a pool of candidate proteins from total cell lysates and all 4 sub-cellular fractions of MMB-biotin treated primary AML cells. To exclude non-specific binding events, pull-down products from biotin control-treated total cell lysates were also identified through the same LC-MS/MS platform. The final list of 312 proteins (Supplemental Table 1) contains targets that are uniquely identified in the MMB-biotin treated cells, but not biotin control treated cells.

Quantitative Real-Time PCR
Total mRNA was isolated with the RNeasy plus mini kit (Qiagen) according to manufacturer’s instructions. mRNA purity and quantity were determined with NanoDrop (Thermo). mRNA samples were reverse transcribed into cDNA using the iScript One-Step RT-PCR Kit (Bio-Rad). Quantitative Real-Time PCR was performed with LightCycler480 real-time PCR using LightCycler 480 SYBR Green I Master Mix reagent (Roche). Primer sequences used for QPCR analysis are listed in Supplemental Table 8.

RNA-seq
The TruSeq RNA Sample Preparation Kit V2 (Illumina) was used for next generation sequencing library construction per manufacturer’s protocols. Amplicons are approximately 200-500bp in size. The amplified libraries were hybridized to the Illumina single end flow cell and amplified using the cBot (Illumina). Single end reads of 100 nt were generated for each sample and aligned to the organism specific reference genome. Raw reads generated from the Illumina HiSeq2500 sequencer were de-multiplexed using configurecl2fastq.pl version 1.8.4. Quality filtering and adapter removal was performed using Trimmomatic version 0.32 with the following parameters: “SLIDINGWINDOW:4:20 TRAILING:13 LEADING:13 ILLUMINACLIP:adapters.fasta:2:30:10 MINLEN:15”. Processed/cleaned reads were then mapped to the UCSC hg19 genome build with SHRIMP version 2.2.3 with the following setting: “--qv-offset 33 --all-contigs”.

Extraction of metabolites and Metabolomic Analysis
DMSO or PTL-treated cells were pelleted and immediately extracted in ice-cold lysis/extraction buffer (methanol:acetonitrile:water 5:3:2) at 2 million cells per ml. Samples were then agitated at 4°C for 30 min and centrifuged at 10,000xg for 15 min at 4°C. Protein and lipid pellets were discarded, while supernatants were stored at -80°C prior to metabolomic analyses. Metabolomic analyses were performed as previously reported (57,58). 20 ml of each sample was injected into an UHPLC system (Ultimate 3000, Thermo) and separated during a 3 min isocratic gradient at 250 ml/min (mobile phase: 5% acetonitrile, 95% 18 mΩ H2O, 0.1% formic acid) on a Kinetex C18 column (150x1 mm i.d., 1.7 mm particle size – Phenomenex). The UHPLC system was coupled online with a QExactive system (Thermo), scanning in Full MS mode (2 mscans) at 70,000 resolution in the 60-900 m/z range, 4kV spray voltage, 15 sheath gas and 5 auxiliary gas, operated either in negative and positive ion mode. Calibration was performed before each analysis against positive or negative ion mode calibration mixes (Piercenet – Thermo) to ensure sub ppm error on the intact mass. Upon conversion of .raw files into .mzXML format using MassMatrix, metabolite and isotopologue assignments were performed using the software Maven1, following the rationale described by Buescher et al (38). Assignments were further confirmed by chemical formula determination from isotopic patterns and accurate intact mass and retention times against over 650 standards, including commercially available glycolytic and Krebs cycle intermediates, amino acids, glutathione intermediates and nucleoside phosphates (Sigma, IROATech).

Cell viability assay
After each treatment, cells were washed with ice-cold FACS buffer (PBS with 0.5% FBS) and then stained for 15 min at 4°C in FACS buffer containing antibodies against human CD34 (BD). After staining, cells were washed with ice-cold FACS buffer, and then stained in Annexin-V binding buffer (10 mM HEPES/NaOH pH 7.4; 140 mM NaCl; 2.5 mM CaCl2) containing Annexin-V
Rational drug regimen to eradicate AML stem cells

*(BD) and DAPI (Sigma). In some cases, viability was measured by Annexin-V and 7-ADD (Sigma) staining instead. For cells which did not need CD34 staining, after FACS buffer wash, cells were directly stained with viability dyes. Stained cells were analyzed immediately on a LSRII flow cytometer (BD). Viable cells were scored as Annexin-V and DAPI/7-AAD double negative cells.

**Colony forming unit (CFU) assay**
Primary AML cells and normal MNCs were plated in Human Methylcellulose Complete Media (R&D Systems) at $2.5 \times 10^4$ cells per ml and $5 \times 10^4$ cells per ml, respectively. Colonies were counted and scored after 3 weeks of culture at $37^\circ C$, 5% CO$_2$ incubator.

**Ex vivo treatment and Xenograft engraftment assay**
Overnight treated cells were washed and resuspended in FACS buffer at 50 million cells per ml. About 5 million cells per mouse were injected intra-peritoneally (IP) into NSG mice. At about 7-10 weeks post injection, all groups of mice were sacrificed, their bone marrow cells were harvested, and the total percentage of human CD45+ cells was quantified to determine engraftment potential.

**Silver stain and Western blot**
Silver stain was performed using the Pierce Silver Stain Kit (Thermo). After gel transfer, blots were probed with SA-HRP (Thermo). To detect specific antigens, blot were probed with primary antibodies against IKB$\alpha$, Histone H3, Caspase-3, phosphor-p65-Ser536 (Cell Signaling), IL6R$\alpha$, Tubulin, Actin (Santa Cruz), and HMOX1 (Stressgen) on a shaker at 4$^\circ C$, overnight, followed by 2 hours of room temperature incubation with HRP-conjugated secondary antibodies (Santa Cruz). Chemo-luminescence was recorded using the automated Gel Doc XR+ system (Bio-Rad) or X-ray films (Thermo).

**NADPH/NADP+ measurement**
Quantification of NADPH and NADP+ were performed using the NADP/NADPH-Glo Assay Kit (Promega) according to the manufacture’s protocol.

**Glutathione measurement**
Glutathione quantification was performed using the Glutathione Colorimetric Assay Kit (Biovision) according to the manufacture’s protocol.

**Cellular ROS measurement**
Cells were incubated at 37 $^\circ$C in ROS-staining buffer (PBS+2%FBS) containing the CM-H$_2$DCFDA probe (1uM) (Life technologies) for 30 min. After incubation, cells were washed 2 times with ROS-staining buffer before FACS analysis.

**NF-$\kappa$B EMSA assay**
NF-$\kappa$B DNA binding activity in cell nuclear extracts was measured using electrophoretic mobility shift assay as previously described (59). Briefly, an oligonucleotide corresponding to the consensus sequence (5’-AGTTGAGGGGACTTTC CCAGGC-3’) to NF-$\kappa$B was radiolabeled and incubated with nuclear extract. The DNA-protein complexes were resolved in 5% native PAGE in low ionic strength buffer. The gels were dried, and protein binding was visualized by autoradiography.

**Pathway analysis using IPA, GSEA, and iPAGE**
The list of 312 PTL-binding targets (Supplemental Table 1) and the list of 2114 significantly dysregulated genes induced by PTL (Supplemental Table 3) were used to perform the IPA (Qiagen) core analysis with default settings. GSEA (http://www.broad.mit.edu/gsea/) analysis (60) was run on the c2.cp.kegg.v5.0 or the c2.cp.reactome.v5.0 genesets, using 1000 permutations of the genesets. Gene sets with less than 15 genes or more than 500 genes were excluded. Gene sets with a FDR $\leq 0.25$ and a nominal $p \leq 0.05$ were considered significant. iPAGE method was used to identify over and under-represented pathways (42) using RNA-seq datasets of PDT-induced AML samples. Briefly in iPAGE, we quantized continuous expression data into equally populated bins. iPAGE then calculated the mutual information (MI) between a vector of expression values and a binary vector of pathway memberships for every pathway. The significance of the calculated MI values was then assessed through a randomization-based statistical test. We then used hyper-geometric distribution to determine the level with which the significantly informative pathways are over-represented or under-represented.
under-represented in each expression bin or cluster. We used the resulting p values to draw a heat map, in which rows represent significant pathways and columns correspond to expression bin/clusters. In the heat map, red entries correspond to pathway over-representations, while blue entries correspond to under-representations.

Statistics
Unless otherwise indicated, statistical analyses were performed using two-tailed (non-directional), type three (unequal variance) student’s t-test. A p value of less than 0.05 indicates significance.

Study approval
All experimental materials and procedures involving human and animals in this study were reviewed and approved by University of Colorado under the approval number of 12-0173 and B-103413(09)1E, respectively.
ACKNOWLEDGEMENTS

The work described in this manuscript was supported by Leukemia and Lymphoma Society grants TRP 6230-11 and 6133-12 (CTJ), NIH grants R01 CA158275 (PAC), and R33 CA183685 (KCH).

CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

AUTHOR CONTRIBUTIONS

SP and CTJ designed the study, analyzed the data, and wrote the paper. SP carried out MMB-biotin pull down assay, targets identification, and IPA analysis. FKH performed the LC-MS/MS characterization. SP, AD, TN, and KCH carried out the metabolomic assay and data analyses. SP carried out the glutathione, ROS, QPCR, WB, and NADPH assays related to Fig. 3. SP and MM carried out the NFkB EMSA assay, p-P65 WB, and QPCR assays to determine the activity of PDT to NFkB signaling and ER stress. SP and JMA carried out the RNAseq analysis. SP carried out the in vitro toxicity assay, CFU assay, and engraftment assay to determine the selective antileukemic activity of PDT. BMS, BA, and NK helped engraftment assay and flow cytometry analysis. VKY and SD carried out the iPAGE analysis. JAG, DAP, and CS provided primary AML specimens. PAC provided MMB-biotin.
REFERENCES

1. Salk, J. J., Fox, E. J., and Loeb, L. A. (2010) Mutational heterogeneity in human cancers: origin and consequences. *Annual review of pathology* 5, 51-75
2. Meacham, C. E., and Morrison, S. J. (2013) Tumour heterogeneity and cancer cell plasticity. *Nature* 501, 328-337
3. Hoey, T. (2010) Drug resistance, epigenetics, and tumor cell heterogeneity. *Science translational medicine* 2, 28ps19
4. Catenacci, D. V. (2015) Next-generation clinical trials: Novel strategies to address the challenge of tumor molecular heterogeneity. *Molecular oncology* 9, 967-996
5. Marcucci, G., Haferlach, T., and Dohner, H. (2011) Molecular genetics of adult acute myeloid leukemia: prognostic and therapeutic implications. *J Clin Oncol* 29, 475-486
6. Patel, J. P., Gonen, M., Figueroa, M. E., Fernandez, H., Sun, Z., Racevskis, J., Van Vlierberghe, P., Dolgalev, I., Thomas, S., Aminova, O., Huberman, K., Cheng, J., Viale, A., Socci, N. D., Heguy, A., Cherry, A., Vance, G., Higgins, R. R., Ketterling, R. P., Gallagher, R. E., Litzow, M., van den Brink, M. R., Lazarus, H. M., Rowe, J. M., Luger, S., Ferrando, A., Paietta, E., Tallman, M. S., Melnick, A., Abdel-Wahab, O., and Levine, R. L. (2012) Prognostic relevance of integrated genetic profiling in acute myeloid leukemia. *The New England journal of medicine* 366, 1079-1089
7. Sarry, J. E., Murphy, K., Perry, R., Sanchez, P. V., Secreto, A., Keefer, C., Swider, C. R., Strzelecki, A. C., Cavelier, C., Recher, C., Mansat-De Mas, V., Delabesse, E., Danet-Desnoyers, G., and Carroll, M. (2011) Human acute myelogenous leukemia stem cells are rare and heterogeneous when assayed in NOD/SCID/IL2Rgammac-deficient mice. *The Journal of clinical investigation* 121, 384-395
8. Eppert, K., Takenaka, K., Lechman, E. R., Waldron, L., Nilsson, B., van Galen, P., Metzeler, K. H., Poeppl, A., Ling, V., Beyene, J., Canty, A. J., Danska, J. S., Bohlander, S. K., Buske, C., Minden, M. D., Golub, T. R., Jurisica, I., Ebert, B. L., and Dick, J. E. (2011) Stem cell gene expression programs influence clinical outcome in human leukemia. *Nature medicine* 17, 1086-1093
9. Bonnet, D., and Dick, J. E. (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature medicine* 3, 730-737
10. Jordan, C. T., Guzman, M. L., and Noble, M. (2006) Cancer stem cells. *The New England journal of medicine* 355, 1253-1261
11. Tan, B. T., Park, C. Y., Ailles, L. E., and Weissman, I. L. (2006) The cancer stem cell hypothesis: a work in progress. *Laboratory investigation; a journal of technical methods and pathology* 86, 1203-1207
12. Dick, J. E. (2008) Stem cell concepts renew cancer research. *Blood* 112, 4793-4807
13. Jordan, C. T. (2010) Targeting myeloid leukemia stem cells. *Science translational medicine* 2, 31ps21
14. Jin, L., Lee, E. M., Ramshaw, H. S., Busfield, S. J., Peoppl, A. G., Wilkinson, L., Guthridge, M. A., Thomas, D., Barry, E. F., Boyd, A. D., Gearing, D. P., Vairo, G., Lopez, A. F., Dick, J. E., and Lock, R. B. (2009) Monoclonal antibody-mediated targeting of CD123, IL-3 receptor alpha chain, eliminates human acute myeloid leukemic stem cells. *Cell stem cell* 5, 31-42
15. Jin, L., Hope, K. J., Zhai, Q., Smadja-Joffe, F., and Dick, J. E. (2006) Targeting of CD44 eradicates human acute myeloid leukemic stem cells. *Nature medicine* 12, 1167-1174
16. Lagadinou, E. D., Sach, A., Callahan, K., Rossi, R. M., Neering, S. J., Minhajuddin, M., Ashton, J. M., Pei, S., Grose, V., O'Dwyer, K. M., Liesveld, J. L., Brookes, P. S., Becker, M. W., and Jordan, C. T. (2013) BCL-2 inhibition targets oxidative phosphorylation and selectively eradicates quiescent human leukemia stem cells. *Cell stem cell* 12, 329-341
17. Zhao, C., Chen, A., Jamieson, C. H., Fereshteh, M., Abrahamsson, A., Blum, J., Kwon, H. Y., Kim, J., Chute, J. P., Rizzieri, D., Munchhoff, M., VanArsdale, T., Beachy, P. A., and Reya, T.
Rational drug regimen to eradicate AML stem cells

(2009) Hedgehog signalling is essential for maintenance of cancer stem cells in myeloid leukaemia. Nature 458, 776-779

18. Guzman, M. L., Rossi, R. M., Neelakantan, S., Li, X., Corbett, C. A., Hassane, D. C., Becker, M. W., Bennett, J. M., Sullivan, E., Lachowicz, J. L., Vaughan, A., Sweeney, C. J., Matthews, W., Carroll, M., Liesveld, J. L., Crooks, P. A., and Jordan, C. T. (2007) An orally bioavailable parthenolide analog selectively eradicates acute myelogenous leukemia stem and progenitor cells. Blood 110, 4427-4435

19. Kikushige, Y., Shima, T., Takayanagi, S., Urata, S., Miyamoto, T., Iwasaki, H., Takenaka, K., Teshima, T., Tanaka, T., Inagaki, Y., and Akashi, K. (2010) TIM-3 is a promising target to selectively kill acute myeloid leukemia stem cells. Cell stem cell 7, 708-717

20. Ghantous, A., Sinjab, A., Herceg, Z., and Darwiche, N. (2013) Parthenolide: from plant shoots to cancer roots. Drug discovery today 18, 894-905

21. Mathema, V. B., Koh, Y. S., Thakuri, B. C., and Sillanpaa, M. (2012) Parthenolide, a sesquiterpene lactone, expresses multiple anti-cancer and anti-inflammatory activities. Inflammation 35, 560-565

22. Guzman, M. L., Rossi, R. M., Karnischky, L., Li, X., Peterson, D. R., Howard, D. S., and Jordan, C. T. (2005) The sesquiterpene lactone parthenolide induces apoptosis of human acute myelogenous leukemia stem and progenitor cells. Blood 105, 4163-4169

23. Basu, A., Bodycombe, N. E., Cheah, J. H., Price, E. V., Liu, K., Schaefer, G. I., Ebright, R. Y., Stewart, M. L., Ito, D., Wang, S., Bracha, A. L., Liefeld, T., Wawer, M., Gilbert, J. C., Wilson, A. J., Stransky, N., Kryukov, G. V., Dankic, V., Barretina, J., Garraway, L. A., Hon, C. S., Munoz, B., Bittker, J. A., Stockwell, B. R., Khabele, D., Stern, A. M., Clemons, P. A., Shamji, A. F., and Schreiber, S. L. (2013) An interactive resource to identify cancer genetic and lineage dependencies targeted by small molecules. Cell 154, 1151-1161

24. Zhang, Q., Lu, Y., Ding, Y., Zhai, J., Ji, Q., Ma, W., Yang, M., Fan, H., Long, J., Tong, Z., Shi, Y., Jia, Y., Han, B., Zhang, W., Qiu, C., Ma, X., Li, Q., Shi, Q., Zhang, H., Li, D., Zhang, J., Lin, J., Li, L. Y., Gao, Y., and Chen, Y. (2012) Guaianolide sesquiterpene lactones, a source to discover agents that selectively inhibit acute myelogenous leukemia stem and progenitor cells. Journal of medicinal chemistry 55, 8757-8769

25. Pei, S., Minhajuddin, M., Callahan, K. P., Balys, M., Ashton, J. M., Neering, S. J., Lagadinou, E. D., Corbett, C., Ye, H., Liesveld, J. L., O'Dwyer, K. M., Li, Z., Shi, L., Greninger, P., Settleman, J., Benes, C., Hagen, F. K., Munger, J., Crooks, P. A., Becker, M. W., and Jordan, C. T. (2013) Targeting aberrant glutathione metabolism to eradicate human acute myelogenous leukemia cells. The Journal of biological chemistry 288, 33542-33558

26. Nasim, S., Pei, S., Hagen, F. K., Jordan, C. T., and Crooks, P. A. (2010) Melampomagnolide B: a new antileukemic sesquiterpene. Bioorg Med Chem 19, 1515-1519

27. Kwok, B. H., Koh, B., Ndubuisi, M. I., Elofsson, M., and Crews, C. M. (2001) The anti-inflammatory natural product parthenolide from the medicinal herb Feverfew directly binds to and inhibits IkappaB kinase. Chem Biol 8, 759-766

28. Garcia-Pineres, A. J., Castro, V., Mora, G., Schmidt, T. J., Strunck, E., Pahl, H. L., and Merfort, I. (2001) Cysteine 38 in p65/NF-kappaB plays a crucial role in DNA binding inhibition by sesquiterpene lactones. The Journal of biological chemistry 276, 39713-39720

29. Bork, P. M., Schmitz, M. L., Kuhnt, M., Escher, C., and Heinrich, M. (1997) Sesquiterpene lactone containing Mexican Indian medicinal plants and pure sesquiterpene lactones as potent inhibitors of transcription factor NF-kappaB. FEBS letters 402, 85-90

30. Ralser, M., Wamelink, M. M., Struyts, E. A., Joppich, C., Krobitsch, S., Jakobs, C., and Lehrach, H. (2008) A catabolic block does not sufficiently explain how 2-deoxy-D-glucose inhibits cell growth. Proceedings of the National Academy of Sciences of the United States of America 105, 17807-17811

31. Hassane, D. C., Sen, S., Minhajuddin, M., Rossi, R. M., Corbett, C. A., Balys, M., Wei, L., Crooks, P. A., Guzman, M. L., and Jordan, C. T. (2010) Chemical genomic screening reveals
synergism between parthenolide and inhibitors of the PI-3 kinase and mTOR pathways. *Blood* **116**, 5983-5990

32. Hassane, D. C., Guzman, M. L., Corbett, C., Li, X., Abboud, R., Young, F., Liesveld, J. L., Carroll, M., and Jordan, C. T. (2008) Discovery of agents that eradicate leukemia stem cells using an in silico screen of public gene expression data. *Blood* **111**, 5654-5662

33. Engreitz, J. M., Daigle, B. J., Jr., Marshall, J. J., and Altman, R. B. (2010) Independent component analysis: mining microarray data for fundamental human gene expression modules. *Journal of biomedical informatics* **43**, 932-944

34. Ma, Q. (2013) Role of nrf2 in oxidative stress and toxicity. *Annual review of pharmacology and toxicology* **53**, 401-426

35. Hipp, M. S., Park, S. H., and Hartl, F. U. (2014) Proteostasis impairment in protein-misfolding and -aggregation diseases. *Trends in cell biology* **24**, 506-514

36. D'Alessandro, A., Amelio, I., Berkers, C. R., Antonov, A., Voussen, K. H., Melino, G., and Zolla, L. (2014) Metabolic effect of TAp63alpha: enhanced glycolysis and pentose phosphate pathway, resulting in increased antioxidant defense. *Oncotarget* **5**, 7722-7733

37. Stincone, A., Prigione, A., Cramer, T., Wamelink, M. M., Campbell, K., Cheung, E., Ollin-Sandoval, V., Gruning, N., Kruger, A., Tauqueer Alam, M., Keller, M. A., Breitenbach, M., Brindle, K. M., Rabinowitz, J. D., and Rasler, M. (2014) The return of metabolism: biochemistry and physiology of the pentose phosphate pathway. *Biological reviews of the Cambridge Philosophical Society*

38. Buescher, J. M., Antoniewicz, M. R., Boros, L. G., Burgess, S. C., Brunengraber, H., Clish, C. B., DeBerardinis, R. J., Feron, O., Frezza, C., Ghesquiere, B., Gottlieb, E., Hiller, K., Jones, R. G., Kamphorst, J. J., Kabbe, R. G., Kimmelman, A. C., Locasale, J. W., Lunt, S. Y., Maddocks, O. D., Malloy, C., Metallo, C. M., Meuillet, E. J., Munger, J., Noh, K., Rabinowitz, J. D., Rasler, M., Sauer, U., Stephanopoulos, G., St-Pierre, J., Tennant, D. A., Wittmann, C., Vander Heiden, M. G., Vazquez, A., Voussen, K., Young, J. D., Zamboni, N., and Fendt, S. M. (2015) A roadmap for interpreting C metabolite labeling patterns from cells. *Current opinion in biotechnology* **34**, 189-201

39. Dalle-Donne, I., Rossi, R., Colombo, G., Giustarini, D., and Milzani, A. (2009) Protein S-glutathionylation: a regulatory device from bacteria to humans. *Trends in biochemical sciences* **34**, 85-96

40. Dalle-Donne, I., Aldini, G., Carini, M., Colombo, R., Rossi, R., and Milzani, A. (2006) Protein carbonylation, cellular dysfunction, and disease progression. *Journal of cellular and molecular medicine* **10**, 389-406

41. Aiken, C. T., Kaake, R. M., Wang, X., and Huang, L. (2011) Oxidative stress-mediated regulation of proteasome complexes. *Molecular & cellular proteomics : MCP* **10**, R110 006924

42. Goodarzi, H., Elemento, O., and Tavazoie, S. (2009) Revealing global regulatory perturbations across human cancers. *Molecular cell* **36**, 900-911

43. Oyadomari, S., and Mori, M. (2004) Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell death and differentiation* **11**, 381-389

44. Samudio, I., Harmanecy, R., Fiegl, M., Kantarjian, H., Konopleva, M., Korchin, B., Kalluarchchi, K., Borrmann, W., Duvvuri, S., Taegtmeyer, H., and Andreiff, M. (2010) Pharmacologic inhibition of fatty acid oxidation sensitizes human leukemia cells to apoptosis induction. *The Journal of clinical investigation* **120**, 142-156

45. Krause, D. S., Fackler, M. J., Civin, C. I., and May, W. S. (1996) CD34: structure, biology, and clinical utility. *Blood* **87**, 1-13

46. Spaans, S. K., Weusthuis, R. A., van der Oost, J., and Kengen, S. W. (2015) NADPH-generating systems in bacteria and archaea. *Frontiers in microbiology* **6**, 742

47. Sallmyr, A., Fan, J., Datta, K., Kim, K. T., Grosu, D., Shapiro, P., Small, D., and Rassool, F. (2008) Internal tandem duplication of FLT3 (FLT3/ITD) induces increased ROS production, DNA damage, and misrepair: implications for poor prognosis in AML. *Blood* **111**, 3173-3182
Rational drug regimen to eradicate AML stem cells

48. Sallmyr, A., Fan, J., and Rassool, F. V. (2008) Genomic instability in myeloid malignancies: increased reactive oxygen species (ROS), DNA double strand breaks (DSBs) and error-prone repair. *Cancer Lett* **270**, 1-9

49. Er, T. K., Tsai, S. M., Wu, S. H., Chiang, W., Lin, H. C., Lin, S. F., Wu, S. H., Tsai, L. Y., and Liu, T. Z. (2007) Antioxidant status and superoxide anion radical generation in acute myeloid leukemia. *Clinical biochemistry* **40**, 1015-1019

50. Morgan, M. J., and Liu, Z. G. (2011) Crosstalk of reactive oxygen species and NF-kappaB signaling. *Cell research* **21**, 103-115

51. Guzman, M. L., Neering, S. J., Upchurch, D., Grimes, B., Howard, D. S., Rizzieri, D. A., Luger, S. M., and Jordan, C. T. (2001) Nuclear factor-kappaB is constitutively activated in primitive human acute myelogenous leukemia cells. *Blood* **98**, 2301-2307

52. Hartl, F. U., Bracher, A., and Hayer-Hartl, M. (2011) Molecular chaperones in protein folding and proteostasis. *Nature* **475**, 324-332

53. Sahasrabuddhe, A. A., and Elenitoba-Johnson, K. S. (2015) Role of the ubiquitin proteasome system in hematologic malignancies. *Immunological reviews* **263**, 224-239

54. Walter, P., and Ron, D. (2011) The unfolded protein response: from stress pathway to homeostatic regulation. *Science* **334**, 1081-1086

55. Dai, C., Whitesell, L., Rogers, A. B., and Lindquist, S. (2007) Heat shock factor 1 is a powerful multifaceted modifier of carcinogenesis. *Cell* **130**, 1005-1018

56. Jhaveri, K., Taldone, T., Modi, S., and Chiosis, G. (2012) Advances in the clinical development of heat shock protein 90 (Hsp90) inhibitors in cancers. *Biochimica et biophysica acta* **1823**, 742-755

57. Nemkov, T., D'Alessandro, A., and Hansen, K. C. (2015) Three-minute method for amino acid analysis by UHPLC and high-resolution quadrupole orbitrap mass spectrometry. *Amino acids*

58. D'Alessandro, A., Nemkov, T., Kelher, M., West, F. B., Schwindt, R. K., Banerjee, A., Moore, E. E., Silliman, C. C., and Hansen, K. C. (2015) Routine storage of red blood cell (RBC) units in additive solution-3: a comprehensive investigation of the RBC metabolome. *Transfusion* **55**, 1155-1168

59. Minhajuddin, M., Fazal, F., Bijli, K. M., Amin, M. R., and Rahman, A. (2005) Inhibition of mammalian target of rapamycin potentiates thrombin-induced intercellular adhesion molecule-1 expression by accelerating and stabilizing NF-kappa B activation in endothelial cells. *Journal of immunology* **174**, 5823-5829

60. Subramanian, A., Tamayo, P., Mootha, V. K., Mukherjee, S., Ebert, B. L., Gillette, M. A., Paulovich, A., Pomeroy, S. L., Golub, T. R., Lander, E. S., and Mesirov, J. P. (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 15545-15550
FIGURE LEGENDS

Figure 1. Characterization of PTL’s proteomic interactome in primary AML cells.
(A) Chemical structure of parthenolide. (B) Chemical structure of MMB-biotin. (C) Silver stain of primary AML total cell lysates and sub-cellular fractions on a SDS-PAGE gel. (D) Western blot showing the purity of sub-cellular fractions. (E) Western blot showing the SA pull-down products from total lysates and all sub-cellular fractions, probed with streptavidin-HRP. (F) A schematic diagram illustrating the methodology used to compare the proteomic interactome and transcriptomic signature of PTL in primary AML cells. (G) Number of PTL-binding targets within top 5 most perturbed signaling pathways induced by PTL in primary AML cells. p-values were calculated by IPA analysis to test if a particular pathway is significantly represented by the proteomic interacome of PTL.

Figure 2. Metabolomic analyses reveal increased PPP activity for NADPH production in PTL-treated AML cells.
(A) Schematic diagram showing the workflow used to characterize PTL-induced metabolomic changes in primary AML cells. Results from the uniform labeled U-13C6-glucose (B) and the dual-labeled 13C1,2-glucose (C) labeling experiments showing quantification of 13C-labeled metabolites in glycolysis, PPP, and TCA cycle in primary AML cells following DMSO or 7.5 μM PTL treatment. Data represent mean ± SD (n=3). For each metabolite, “M+x” indicates specific isotopologues of a metabolite labeled with x numbers of the heavy 13C isotopic carbons. A circle diagram is also given to each metabolite to illustrate its carbon frame labeled with different numbers of 13C carbons (red solid circles represent heavy 13C, clear open circles represent 12C). (D) Quantification of NADPH and NADPH/NADP+ ratio in primary AML cells treated with DMSO control or 7.5 μM PTL using a bioluminescent assay. Data represent mean ± SD (n=3).

Figure 3. The PDT regimen depletes glutathione, induces ROS without activating Nrf2-mediated oxidative stress response and NADPH production.
(A) A simplified diagram illustrating the basic rationale of the triple drug regimen PDT. (B) Amount of total glutathione in primary AML cells after 4 hours of treatment. (C) Intracellular ROS level in primary AML cells after each treatment, quantified by the CM-H2DCFDA dye. (D) QPCR data showing fold changes of mRNA expression induced by 7.5 μM PTL or the PDT regimen. Each line represents an individual AML specimen (n=4). (E) Western blot data showing protein expression of HMOX1 following various treatments for 6 h. (F) QPCR data showing fold changes of mRNA expression induced by 7.5 μM PTL or the PDT regimen. Bars represent mean ± SD (n=4). (G) Quantification of NADPH level in primary AML cells following various treatments. Bars represent mean ± SD (n=3).

Figure 4. The PDT regimen does not inhibit NF-κB but induces strong protein misfolding stress in primary AML cells.
(A) NF-κB electrophoretic mobility shift assay (EMSA) of primary AML cells following various treatments for 6 hours. Intensity of EMSA signal in each lane was quantified and normalized to the untreated (UNT) group and expressed as relative intensity values below each lane. (B) Western blot showing level of phospho-p65 (Ser 536) in primary AML cells following various treatments for 6 hours. (C) QPCR results showing mRNA expression of NF-κB downstream gene IL6 in primary AML cells following various treatments for 6 hours. (D) GSEA enrichment plots showing up-regulation of both KEGG_PROTEASOME and REACTOME_UNFOLDED_PROTEIN_RESPONSE gene sets in AML cells treated with the PDT regimen. (E) Heat maps showing up-regulation of genes related to chaperone function, ER stress response, and proteasome activity. Fold change (FC) and standard deviation (SD) are given for each gene (n=4). Red indicates increase. Blue indicates decrease. (F) QPCR data showing expression of CHOP mRNA in primary AML cells following various treatments for 6 h. In all sub panels, unless explicitly stated, the doses used in all drug combination including the PDT regimen are 2.5 μM,
0.1125 mg/ml, and 2.5 ug/ml for PTL, 2DG, and TEM, respectively. In (C) and (F), bars represent mean ± SD (n=3).

Figure 5. The PDT regimen effectively eradicates primary AML.  
(A) Viability of 10 primary AML specimens after being treated with various drugs alone or in combination for 24 h. Data are plotted as % of untreated. Bars represent mean ± SD. (B) Viability of 5 PTL-resistant primary AML specimens after being treated with 7.5 uM PTL or the PDT regimen. Data are plotted as % of untreated. Bars represent mean ± SD. (C) Number of colonies in methylcellulose culture per 100K primary AML cells treated with the PDT regimen or 7.5 uM PTL. Bars represent mean ± SD. In all drug combinations including the PDT regimen, PTL, 2DG, and TEM was used at 2.5 uM, 0.1125 mg/ml, and 2.5 ug/ml, respectively.

Figure 6. The PDT regimen is not toxic to normal hematopoietic cells.  
(A) Viability of total normal MNC cells after being treated with various drugs alone or in combination for 24 h. Data are plotted as % of untreated. Bars represent mean ± SD (3 individual MNC specimens). (B) Representative flow cytometry plots showing the impact of PDT treatment to both total MNCs and CD34+ MNCs. Viable MNCs were determined by double negative staining of 7-AAD and Annexin V. Percentage of CD34+ cells were quantified within the viable MNCs. (C) Viable CD34+ normal MNCs after being treated with various drugs alone or in combination for 24h. Data are normalized to untreated control. Bars represent mean ± SD (3 individual MNC specimens). (D) Number of colonies in methylcellulose culture per 1 e6 normal MNCs treated with or without the PDT regimen. Bars represent mean ± SD (n=3). In all different drug combinations including the PDT regimen, PTL, 2DG, and TEM was used at 2.5 uM, 0.1125 mg/ml, and 2.5 ug/ml, respectively.

Figure 7. The PDT regimen preferentially targets LSCs but spares HSCs.  
(A) Engraftment potential of AML cells treated with various drug combinations in NSG mice. Each dot represents an individual mouse. Lines represent mean ± SD (n=10). (B) Engraftment potential of normal MNCs treated with or without the PDT regimen in NSG mice. Each dot represents an individual mouse. Lines represent mean ± SD (n=10). In all sub panels, the PDT regimen is composed of 2.5 uM PTL, 0.1125 mg/ml 2DG, and 2.5 ug/ml TEM. (C) A working model describing the anti-leukemia mechanism of the PDT regimen.
Fig. 1

A  Parthenolide

B  MMB-biotin

C  Input

Silver Stain

D  WB:
- IKBα (Cyto.)
- IL6Rα (Memb.)
- Histone H3 (Nucl.)
- Tubulin (Cytoske.)

E  SA Pull-down Products

WB: SA-HRP

F  312 targets

Top 5 PTL-induced dysregulated pathways

present in?

G  # of PTL-binding targets vs. p value

- Protein Ubiquitination Pathway
- NRF2-mediated Oxidative Stress Response
- Unfolded protein response
- Aldosterone Signaling in Epithelial Cells
- tRNA Charging
Characterization of PTL-induced metabolomic changes

A

DMSO / 7.5μM PTL

U-13C6-Glu

or

13C12-Glu

Treatment time
0h harvest
4h harvest
6h harvest

Primary AML cells

LC-MS identification

B

PPP

NADPH

Glycolysis

glyceraldehyde 3-phosphate

pyruvate

lactate

aspartate

oxaloacetate

TCA cycle

malate

citrate

C

glyceraldehyde-3-phosphate

(M+1)

D

NADPH

Relative amount

0

5x10^4

1x10^5

2x10^5

DMSO

PTL

p<0.005

NADPH / NADP+

Ratio

0

0.1

0.2

0.3

0.4

0.5

0.6

DMSO

PTL

p<0.005

Primary AML cells
Fig. 3

A) Oxidative Stress

2DG + TEM → Oxidative Stress → Protein misfolding Stress → Cell death

B) Glutathione

Total Glutathione (% of untreated)

C) ROS

UNT

5mM H₂O₂, 30'

7.5μM PTL, 1h

7.5μM PTL, 2h

7.5μM PTL, 4h

7.5μM PTL, 6h

7.5μM PTL, 8h

PDT, 1h

PDT, 2h

PDT, 4h

PDT, 6h

PDT, 8h

CM-H₂DCFDA

MFI

Hours

D) Fold Change

7.5μM PTL induced

PDT induced

Glutathione

HMOX1

Thioredoxin

SLC7A11

SLC3A2

GCLC

GCLM

GSR

H2O2

2DG

TEM

E) WB: HMOX1

ACTIN

F) PPP

Induced Fold Change (log scale)

G6PD

PGD

7.5μM PTL

PDT

7.5μM PTL

PDT

NADPH

RFU

UNT

7.5μM PTL, 5hr

PDT, 1hr

PDT, 2hr

PDT, 4hr

PDT, 6hr

PDT, 8hr
**Fig. 5**

A. **Viable AML cells (n=10)**

B. **PTL-resistant AMLs (n=5)**

C. **CFU assay**

AML #1

AML #5

AML #7

AML #8
Fig. 6

A  Viable total MNCs (n=3)

Viability (% of untreated) vs. treatment conditions.

B  Total MNCs

Untreated vs. PDT conditions

C  Viable CD34+ MNCs (n=3)

Viability (% of untreated) vs. treatment conditions.

D  CFU assay

Comparison of colony numbers between untreated (UNT) and PDT conditions.

Legend:
- PTL: 2.5 μM
- 2DG: 0.1125 mg/ml
- TEM: 2.5 ug/ml
- PTL+2DG
- PTL+TEM
- 2DG+TEM
- PDT

Viability (as % of untreated) for primary NBM cells (n=3).
Fig. 7

A) Primary AML

B) Normal

C) Oxidative Stress Response genes

- Glucose
- NADPH
- GSH
- TXN
- GCLC
- GCLM
- Nrf2
- ROS
- Protein misfolding stress
- Apoptosis

Cytoplasm

Nucleus
Rational Design of a Parthenolide-based Drug Regimen that Selectively Eradicates Acute Myelogenous Leukemia Stem Cells
Shanshan Pei, Mohammad Minhajuddin, Angelo D’Alessandro, Travis Nemkov, Brett M. Stevens, Biniam Adane, Nabilah Khan, Fred K. Hagen, Vinod K. Yadav, Subhajyoti De, John M. Ashton, Kirk C. Hansen, Jonathan A. Gutman, Daniel A. Pollyea, Peter A. Crooks, Clayton Smith and Craig T. Jordan

J. Biol. Chem. published online August 29, 2016

Access the most updated version of this article at doi: 10.1074/jbc.M116.750653

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2016/08/29/M116.750653.DC1
Rational design of a parthenolide-based drug regimen that selectively eradicates acute myelogenous leukemia stem cells.

Shanshan Pei, Mohammad Minhajuddin, Angelo D’Alessandro, Travis Nemkov, Brett M. Stevens, Biniam Adane, Nabilah Khan, Fred K. Hagen, Vinod K. Yadav, Subhajyoti De, John M. Ashton, Kirk C. Hansen, Jonathan A. Gutman, Daniel A. Pollyea, Peter A. Crooks, Clayton Smith, and Craig T. Jordan

The University of Arkansas for Medical Sciences and Dr. Peter A. Crooks have financial interests in melampomagnolide B (MMB) and structurally related analogs discussed in this manuscript. These financial interests have been reviewed and approved in accordance with the university’s conflict of interest policies.