Phenothiazines induce PP2A-mediated apoptosis in T cell acute lymphoblastic leukemia

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T cell acute lymphoblastic leukemia (T-ALL) is an aggressive cancer that is frequently associated with activating mutations in NOTCH1 and dysregulation of MYC. Here, we performed 2 complementary screens to identify FDA-approved drugs and drug-like small molecules with activity against T-ALL. We developed a zebrafish system to screen small molecules for toxic activity toward MYC-overexpressing thymocytes and used a human T-ALL cell line to screen for small molecules that synergize with Notch inhibitors. We identified the antipsychotic drug perphenazine in both screens due to its ability to induce apoptosis in fish, mouse, and human T-ALL cells. Using ligand-affinity chromatography coupled with mass spectrometry, we identified protein phosphatase 2A (PP2A) as a perphenazine target. T-ALL cell lines treated with perphenazine exhibited rapid dephosphorylation of multiple PP2A substrates and subsequent apoptosis. Moreover, shRNA knockdown of specific PP2A subunits attenuated perphenazine activity, indicating that PP2A mediates the drug’s anti-leukemic activity. Finally, human T-ALLs treated with perphenazine exhibited suppressed cell growth and dephosphorylation of PP2A targets in vitro and in vivo. Our findings provide a mechanistic explanation for the recurring identification of phenothiazines as a class of drugs with anticancer effects. Furthermore, these data suggest that pharmacologic PP2A activation in T-ALL and other cancers driven by hyperphosphorylated PP2A substrates has therapeutic potential.

Introduction

T cell acute lymphoblastic leukemia (T-ALL) is an aggressive cancer affecting mainly adolescents and young adults. Intensified treatment regimens have improved outcomes, but patients who fail conventional therapy have a dismal prognosis, and T-ALL remains fatal in 20% of children and more than 50% of adults (1–3). New therapies are desperately needed for patients in these poor-prognosis groups.

One of the most common genetic aberrations in T-ALL is activating mutations in NOTCH1 (4), a transmembrane receptor that is converted to a transcriptional activator through a series of proteolytic cleavage events, the last of which is carried out by the intramembranous protease γ-secretase (5). γ-Secretase cleavage allows the intracellular domain of NOTCH1 (ICN1) to translocate to the nucleus and form a transcriptional activation complex. The mutations in NOTCH1 that occur in T-ALL variously stimulate NOTCH1 proteolysis and generation of ICN1 or decrease ICN1 turnover, thereby enhancing expression of ICN1 target genes. One of the most important direct targets of ICN1 in the context of T-ALL cells is MYC (6–8); indeed, enforced expression of MYC can rescue some human T-ALL cell lines from NOTCH1 inhibition (6, 9) and MYC transgenes can drive T-ALL development in mouse (10) and zebrafish (11, 12) models. However, NOTCH1 activation and MYC overexpression are not sufficient for T-ALL development, indicating that establishment and maintenance of T-ALL depends on additional cooperative genetic or epigenetic events that dysregulate other signaling pathways. Indeed, activating NOTCH1 mutations are found together with a diverse collection of other recurrent mutations in human T-ALL (13). Among the most frequent are mutations that activate the PI3K/AKT signaling pathway (14), which has a complex interrelationship with NOTCH signaling in T-ALL cells (15) and is another promising therapeutic target in this disease (16). Repurposing of FDA-approved drugs is an attractive approach to drug discovery, as it can in principle enable rapid translation to the clinic. Using 2 complementary screens, we identified phenothiazine...
azines as a class of drugs with NOTCH-independent anti–T-ALL activity. Phenothiazines have been used for over 50 years as neuroleptic-type antipsychotic medications. The antipsychotic effects of phenothiazines correlate with their ability to block dopamine receptors, but a broad array of other activities have been described, including antitumor effects. The basis for the antiproliferative activities of phenothiazines are uncertain and have been variously attributed to a number of mechanisms, including inhibition of PKC (17), calmodulin (18), PI3K/AKT signaling (18–21), and cancer stem cell activity (22).

Using quantitative mass spectrometry to analyze drug-protein binding proteome-wide, we identified the tumor suppressive serine/threonine phosphatase protein phosphatase 2A (PP2A) as a new phenothiazine target (Alex Kentsis and James E. Bradner, personal correspondence). Phenothiazines stimulate rapid dephosphorylation of multiple PP2A targets implicated in tumor cell growth and survival in cells, and have antitumor effects on T-ALL cells in vitro and in vivo. Our findings provide a likely explanation for the recurrent discovery of phenothiazines in screens for compounds with anticancer effects, point to new rational drug combinations for consideration in treatment of NOTCH-driven cancers such as T-ALL, and provide additional impetus for development and testing of PP2A activators in a wide variety of cancers.

Results

Zebrafish screen for small molecules that are toxic to MYC-overexpressing thymocytes. We previously developed a zebrafish model of MYC-induced T-ALL that closely resembles the human disease morphologically and by gene expression (11, 12, 23), and we wanted to exploit this model for in vivo drug discovery. We thus developed a fluorescence-based screen that was designed to identify small molecules that are selectively cytotoxic to MYC-overexpressing thymocytes. Heterozygous zebrafish carrying a tandem rag2:MYC-ER;mitfa transgene (created by cointegration of rag2:MYC-ER and wild-type mitfa transgenes at the same genomic locus) were mated to rag2:dsRed2 homozygous animals, generating rag2:MYC-ER–positive or –negative embryos that both express rag2:dsRed2. All crosses were performed in a nacre pigment–mutant background in which melanophores were absent due to homozygous mitfa-inactivating mutations (24). The rescue of the pigmentation defect in fish carrying the rag2:MYC-ER;mitfa transgene allowed us to distinguish MYC-ER–positive and –negative zebrafish at 3 days post-fertilization (dpf) (Figure 1A). At 3 dpf, zebrafish larvae were arrayed into 96-well plates (3 larvae/well) and incubated with 12.5 μM drug and 4-hydroxytamoxifen to activate the MYC-ER fusion protein. Four days later, microscopy was performed to assay thymic fluorescence, which was scored as 3 (normal), 2 (intermediate), 1 (weak), or 0 (no fluorescence) (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI65093DS1). Any compound that induced complete loss of fluorescence in 1 fish or weak fluorescence in 2 of the 3 larvae per well, was considered a hit in this primary screen. Compounds that induced either death of larvae or generalized developmental abnormalities were excluded from further analysis. Four libraries of bioactive small molecules comprising 4,880 FDA-approved drugs, drug-like small molecules, and natural products were screened in this fashion.
Although we did not identify compounds that were selectively toxic to MYC-overexpressing thymocytes, multiple compounds with activity against both MYC-positive and -negative thymocytes were identified. Among these hits were numerous glucocorticoids, which are well known inducers of thymocyte apoptosis, as well as conventional chemotherapeutics that induce DNA damage (alkylating agents, topoisomerase inhibitors) (Figure 1B and Supplemental Table 1). Many of these compounds are mainstays of contemporary T-ALL therapy, thus validating the utility of our approach. A number of novel compounds were also identified, including perphenazine (PPZ), an FDA-approved phenothiazine antipsychotic (Figure 1, B and C, and Supplemental Table 1). Secondary screening in zebrafish larvae (n = 6 larvae per concentration) confirmed that PPZ is active against MYC-overexpressing thymocytes in a dose-dependent manner (Figure 1D).

**Human T-ALL screen for small molecules that synergize with γ-secretase inhibitors.** In parallel, we conducted a cell-based screen with the Broad Institute bioactives chemical library collection (3,194 compounds) to identify drugs that synergistically suppress the growth of T-ALL cells in combination with gamma-secretase inhibitors (GSI). Roughly two-thirds of the small molecules in this collection (n = 2108) overlap with compounds screened in the zebrafish. The screen was conducted with KOPT-K1 cells, a NOTCH1-dependent T-ALL cell line that overexpresses the transcription factor TAL1, a feature associated with a relatively poor prognosis in this disease. We used the combination of GSI and dexamethasone, a known
synergistic combination (25), as a positive control. We initially performed isobologram analysis to select concentrations of GSI (compound E, 100 nM) and dexamethasone (200 nM) that have a synergistic growth inhibitory effect on KOPT-K1 cells (Supplemental Figure 2) and then used these concentrations of drugs in screen optimization pilot studies. Under the final conditions selected, GSI versus GSI plus dexamethasone produced a Z score of 0.74 (Supplemental Figure 3); for point of reference, screens with Z scores greater than 0.5 are taken as having a high probability of detecting true positives.

We then screened the Broad Institute bioactives library in KOPT-K1 cells according to the strategy shown in Suppemental Figure 4. In brief, GSI/bioactive-compound interaction was assessed independently scoring the effects of GSI plus each bioactive compound alone (Figure 2A) and the effects of each bioactive compound alone (Figure 2B). To identify compounds that synergize with GSI, we looked at the data using 2 different approaches. First, we com-
to regulate immune function (28). However, the structurally unrelat-
ded dopamine inhibitors haloperidol, domperidone, and clozapine
were not toxic to Myc-overexpressing thymocytes, had no effect on
T-ALL progression in zebrafish, and did not affect human KOPT-K1
T-ALL cell growth (data not shown). Conversely, promethazine, a
phenothiazine that does not inhibit dopaminergic signaling (29),
decreased the growth of zebrafish T-ALLs and human KOPT-K1
T-ALL cells (Figure 3B and Figure 4A). Taken together, these data
strongly suggest that inhibition of dopaminergic signaling is unre-
lated to the antileukemic activity of these compounds.

Another reported activity of phenothiazines that could abrogate
mitogenic signaling is inhibition of calmodulin (30), which has
broad roles in calcium-dependent intracellular signaling. However,
we noted that W13, a relatively selective calmodulin inhibitor, is
highly toxic to zebrafish larvae at low nanomolar concentrations
(data not shown), presumably because calmodulin inhibition is
not compatible with survival. Thus, while it is difficult to exclude
some contribution of calmodulin inhibition to the observed
effects of phenothiazines, calmodulin inhibition cannot explain
the activity of phenothiazines in the zebrafish screen.

We were therefore left with the challenge of identifying the molecular
target that was responsible for the activity of phenothi-
azines in our screens. To address this issue, we devised a quantita-
tive mass spectrometry approach, termed activity correlation pro-
teomics, that offers to improve the identification of biologically
relevant targets of pleiotropic drugs. Briefly, we used fluoros-
tagged PPZ and fluorous ligand-affinity chromatography to pull-
down protein targets from KOPT-K1 T-ALL cell lysates labeled
with stable isotopes (SILAC) (31). These protein pull-downs were
done in the presence or absence of competitor phenothiazines
with varying IC_{50} for KOPT-K1 cell growth inhibition, and dis-
placement of individual binding proteins from the fluorous PPZ
matrix was assessed by quantitative mass spectrometry. We then
rank ordered binding proteins based on the correlation between
displacement by drug and the drug’s IC_{50} in KOPT-K1 cells. This
approach identified PPP2R1A as the protein whose binding
affinity correlated most closely with the biologic activities of the
phenothiazines tested (Figure 6, A and B). PPP2R1A is the A
α
scaffolding subunit of the protein phosphatase PP2A, and sub-
sequent work confirmed that PPZ directly binds purified PP2A at
low micromolar concentrations (Alex Kentsis and James E. Brad-
ner, personal communication).

PP2A is a conserved heterotrimeric phosphatase that has many
different isoforms, each comprising a scaffolding A subunit, a vari-
able regulatory B subunit, and a catalytic C subunit (32, 33). Best
known as a tumor suppressor that is targeted by SV40 small t anti-
gen, PP2A is also a target of the oncogenic microRNA miR-19 (34),
which collaborates with activated Notch1 to promote T-ALL devel-
oment. The tumor-suppressive activity of PP2A is believed to be
mediated through its ability to dephosphorylate diverse substrates
implicated in oncogenic signaling, including AKT (35), p70S6K
(36), MYC (37–39), ERK (40, 41), and BAD (42, 43). Thus, if the
antileukemic effects of PP2 and other phenothiazines in T-ALL
are mediated through PP2A, then these drugs should stimulate
PP2A activity. As predicted, incubation of KOPT-K1 cells with PPZ led to rapid dephosphorylation of the PP2A substrates AKT, ERK, p70S6K, MYC, and BAD (Figure 6, C–E), effects that were abrogated by addition of the phosphatase inhibitor okadaic acid (Figure 6D), an inhibitor of PP2A. Total AKT, ERK, p70S6K, and BAD protein levels were unaffected by PPZ, but the levels of MYC protein fell (Figure 6E), in line with prior reports showing that PP2A-mediated dephosphorylation of MYC residue S62 enhances MYC degradation (37). In addition, we noted that the effects of PPZ on PP2A substrates and growth of T-ALL cell lines such as KOPT-K1 were phenocopied by FTY720 (Fingolimod) (Supplemental Figure 6, A and B), an FDA-approved immunomodulatory drug previously shown to activate PP2A at low micromolar concentrations (44, 45). In contrast, the calmodulin inhibitor W13 had no effect on PP2A targets such as p70S6K (Supplemental Figure 6C).

To determine whether PP2A is required for the activity of PPZ, we first used shRNA to knock down the PP2A α scaffolding subunit. PP2A α knockdown impaired the PPZ-induced dephosphorylation of PP2A targets (Figure 7A) and also desensitized KOPT-K1 cells to the growth-suppressive effects of PPZ (Figure 8B). shRNA knock-down of a catalytic PP2A Cα subunit had similar effects (Figure 7, C and D). Taken together, these data strongly suggest that growth-suppressive effects of PPZ on T-ALL cells are mediated through stimulation of PP2A activity.

PPZ activates PP2A and has antileukemic effects on primary human T-ALL cells in vitro and in vivo. With these results in hand, it remained to be determined whether PPZ could inhibit the growth of primary T-ALL cells in vitro and in vivo, and if so, whether antileukemic effects were associated with dephosphorylation of PP2A targets. To address this issue in vitro, we tested a series of primary human T-ALLs (summarized in Supplemental Table 3) that had been expanded by passage through NSG mice for sensitivity to PPZ in a newly described serum-free culture system for T-ALL cells that relies on completely defined medium and feeder cells expressing the Notch ligand DLL-1 (46). We noted that low micromolar levels of PPZ resulted in dephosphorylation of multiple PP2A targets (AKT, p70S6K, and BAD) and impaired growth of all 4 T-ALLs tested under these conditions (Figure 8). Variable sensitivity to GSI and variable interaction between GSI and PPZ was also noted (Figure 8). We also tested an independent series of primary T-ALLs in a second culture system that relies on high levels of human serum and observed similar results, as all lines were growth inhibited by low micromolar concentrations of PPZ (Supplemental Figure 7).

One of the primary human T-ALL lines, hTALL2, grew sufficiently well in the serum-free culture system to permit luciferization with retrovirus, which facilitated drug testing in vivo. This was carried out in mice bearing calcium phosphate scaffolds seeded with human marrow–derived mesenchymal stem cells, an approach that allows for drug testing within a “humanized” microenvironment. Treatment of mice with PPZ (10 mg/kg intraperitoneally daily) caused a significant decrease in growth of hTALL2 cells in the scaffolds (Figure 9A) and in murine spleen (Figure 9B). Importantly, a single dose of PPZ (10 mg/kg) also led to the dephosphorylation of the PP2A targets AKT, p70S6K, and BAD in hTALL2 blasts in vivo (Figure 9C). These results show that PPZ can produce antileukemic effects and stimulate PP2A activity in primary T-ALL lymphoblasts at doses that are tolerated by mice.

**Discussion**

NOTCH1 is a compelling therapeutic target in T-ALL, but the activity of GSIs as single agents in T-ALL preclinical models (47) and clinical trials (48) has been modest, suggesting the need to...
combine Notch pathway inhibitors with other compounds with activity in this disease. We used complementary screens to discover small molecules with Notch-independent anti-T-ALL activity. Most small molecules scoring in both screens were glucocorticoids, providing further support for use of GSIs and glucocorticoids in combination in relapsed/refractory T-ALL (25). A previously unidentified hit emerging from both screens was PPZ, which was shown to suppress the growth of piscine, murine, and human T-ALLs through induction of apoptosis. These effects of PPZ were linked by biochemical, cell biological, and genetic studies to activation of PP2A, a tumor-suppressive protein phosphatase. The ability of PPZ to activate PP2A thus represents the likely mechanism for the previously unexplained ability of phenothiazines to downregulate PI3K/AKT signaling and may explain the recurrent identification of these compounds in unbiased screens for agents with antineoplastic activity (18–53).

Given that signaling via mutated KRAS (54) and receptors such as IL-7R (55, 56) and IGF-R1 (57) synergize with Notch signaling to induce and maintain T-ALL growth, combinations of agents that antagonize downstream effectors of these pathways are attractive therapeutic options in this disease. Indeed, prior work has shown that GSI in combination with TOR inhibitors (9, 47), PI3K inhibitors (58), and MAPK inhibitors (58) have increased activity over GSI alone against T-ALL. PP2A blunts signaling through multiple “nodes” downstream of surface receptors and RAS via dephosphorylation of signaling intermediates such as ERK (40, 41), AKT (35), and p70S6K (36) and can also inhibit growth by downregulating MYC (37–39) and promote apoptosis by dephosphorylating BAD (42, 43). In support of PPZ acting as a PP2A activator, our data show that PPZ treatment affects phosphorylation of each of these PP2A substrates, whereas shRNA knockdown of PP2A catalytic or scaffolding subunits impaired the antileukemic activity of PPZ. Withdrawal of Notch signaling via treatment with GSI or other inhibitors interferes with the growth of T-ALL cells through a number of mechanisms, including downregulation of MYC (6, 8, 59), IL-7R (55, 60), and IGF-R1 (57), and suppression of PI3K/AKT signaling (61). The partially overlapping effects of PP2A stimulation and Notch withdrawal on key mitogenic signaling pathways would predict that drug combinations that hit both targets should have at least additive growth-suppressive effects on Notch-dependent T-ALL cells, consistent with our findings here with GSI and phenothiazines.
Our data suggest that a combination of a GSI and a PP2A activator is an attractive strategy in relapsed/refractory T-ALL, one that would also merit consideration in other Notch-associated tumors, such as mantle cell lymphoma (62), chronic lymphocytic leukemia (63–65), and breast cancer (66), that have failed conventional therapies. A limitation is that PP2Z and most other phenothiazines have marked extrapyramidal side effects, which is a limitation when considering these drugs as therapeutic agents in cancer, and have activities as antipsychotic drugs at low nanomolar concentrations, substantially below the levels that are needed to activate PP2A. It may be possible to alleviate these drawbacks by engineering phenothiazine derivatives that are more potent PP2A activators and yet lack activity on dopamine receptors; promethazine is an example of a phenothiazine with antileukemia activity that does not avidly bind dopamine receptors, suggesting that these 2 activities can be separated. An alternative approach is to use other PP2A activators with more favorable toxicity profiles that are already FDA approved, such as FTY720 (44). FTY720 is a potent inhibitor of the sphingosine-1-phosphate receptor that has activity in preclinical models of a variety of cancers, including chronic lymphocytic leukemia (67), chronic myelogenous leukemia blast crisis (45), B cell acute lymphoblastic leukemia (45), and mantle cell lymphoma (68). However, as with phenothiazines, the levels of FTY720 that are required for PP2A stimulation are in the low micromolar range, making it a less than ideal clinical lead. It is likely that more active and selective PP2A-stimulating compounds will need to be developed if they are to have a robust clinical impact as anticancer drugs. Identification of the PP2A-stimulating effect of phenothiazines provides a new path forward for the development of small molecules with improved on-PP2A and diminished off-target activities.

Methods

Transgenic zebrafish lines. The rag2:MYC-ER;mtf, rag2:dsRed2, rag2:EGFP-MYC, and rag2-EGFP-BCL2 transgenic lines have previously been described (12, 23, 69).

Zebrafish small molecule screen. At 3 dpf, transgenic zebrafish larvae expressing rag2:Myc-ER and rag2:dsRed2 transgenes (12) were arrayed into 96-well plates (3 larvae per well) and exposed to individual small molecules at doses of 12.5 μM for small molecules and 1.25 μM for bioactive lipids, together with 50 μg/ml (129 nm) 4-hydroxytamoxifen to activate the Myc-ER transgene, as well as antibiotics to reduce bacterial overgrowth (25 units/ml penicillin, 25 mg/ml streptomycin, and 5 mg/ml meropenem). Small molecules were from the following small molecule libraries: Lopac 1280 (n = 1280 compounds; Sigma-Aldrich); ICCB known bioactives (n = 480 compounds; Enzo Life Sciences); the Prestwick Chemical Library (n = 1200 compounds; Prestwick Chemical); and the Spectrum Collection (n = 2000 compounds; MicroSource Discovery Systems). After 4 days of treatment, thymic fluorescence in individual larvae was assessed via fluorescence microscopy. Small molecules that induced general toxicity to any zebrafish larvae or that failed conventional therapies. A limited "pinned" with CyBi-Well in duplicate with compound E (100 nM; DMSO) or 7.5 μM PP2Z. In A and C, all cell viability assays were performed in triplicate. (A) Western blot analysis of KOPT-K1 T-ALL cells infected with shRNA specific for GFP (control) or PP2A Aα and treated with the doses of PP2Z shown. (B) Effect of the same shRNAs on KOPT-K1 T-ALL cell viability at day 5 of treatment with control or 5 μM PP2Z. (C) Western blot analysis of KOPT-K1 T-ALL cells infected with shRNA specific for GFP (control) or PP2A Aα and treated with the doses of PP2Z shown. (D) Effect of the same shRNAs on KOPT-K1 T-ALL cell viability at day 5 of treatment with control (DMSO) or 7.5 μM PP2Z. In A and C, cells were treated with PP2Z for 30 minutes.

Figure 7

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screening experiments are available via ChemBank (http://chembank.broadinstitute.org/assays/view-project.htm?id=1000654). To combine the results of the 2 assays quantitatively, we defined a trigonometric scaling function to express our preferences that (a) +GSI composite Z scores be negative, and (b) +GSI composite Z score amplitudes exceed –GSI composite Z score amplitudes; such compounds should fall left of the DMSO-control distribution, but above the main diagonal in a plot of –GSI composite Z versus +GSI composite Z (Figure 2C). Using the ratio of ChemBank composite Z scores (–GSI/+GSI), we defined \( \theta = \arctan(\frac{Z_{-GSI}}{Z_{+GSI}}) \) and expressed our scale factor as \( F = \cos(-\theta)\cos(2\theta) \), with the 2 factors respectively expressing our 2 preferences. As a 2-assay composite score, we take \( -(Z_{+GSI}) \times F \), which clearly discriminates the compounds of interest when plotted against \( Z_{-GSI} \) (Figure 2D). Using an initial cutoff of \( F > 0.5 \), we identified 330 compounds, of which 43 compounds also were statistically distinct from the 2-assay DMSO distribution, modeled as a multivariate normal distribution, when subjected to Holm-Bonferroni multiple hypothesis test correction.

Cell culture. All cells were cultured at 37°C with 5% CO2. Established cell lines were grown in RPMI supplemented with 10% fetal calf serum, glutamine, and penicillin/streptomycin. Primary human T-ALLs were grown in short-term cultures on MS5-DLL1 stromal cells in either high-serum (71) or in serum-free defined conditions (46) as described.

Cell viability assays. Cell viability was measured using the CellTiter-Blue cell viability assay kit (Promega). Cells were plated in the black-walled 96-well plates and treated with chemical compounds. Then 20 \( \mu l \) of CellTiter-Blue reagent was added to wells, each of which contained 100 \( \mu l \) of cells. After 4 hours at 37°C, fluorescence was recorded in a SpectraMax M3 microplate reader (Molecular Devices) to measure resazurin reduction.

Isobologram analysis. To determine whether drugs have synergistic antileukemia cell growth effects, cells were incubated with various concentrations of drugs for 48 hours and then incubated with CellTiter-Blue for an additional 4 hours. Cell numbers were determined by measuring fluorescence at 595 nm in a plate reader. Data were processed and subjected to isobologram analysis (72) using CalcuSyn to determine the combination index (CI) at ED50. A CI of less than 0.7 at ED50 is taken to be indicative of a synergistic interaction.

Cell-cycle analysis. Cells were washed once with cold PBS and fixed with 90% ethanol overnight at –20°C. Cells were then washed once with cold PBS and resuspended in propidium iodide (PI) staining solution (20 \( \mu g/ml \) PI, 200 \( \mu g/ml \) DNAse-free RNAse A, and 0.1% [v/v] Triton X-100 in PBS). Following incubation at 37°C for 15 minutes, the samples were analyzed in a FACSCalibur flow cytometer (BD Biosciences) using Cell Quest software.

Figure 8
PPZ induces rapid dephosphorylation of PP2A targets and inhibits growth of cultured primary human T-ALL cells. (A–D) Western blots showing the effects of incubation for 15 minutes with the indicated doses of PPZ. (E–H) Effects of GSI (compound E, 1 \( \mu M \)) and PPZ (5 \( \mu M \)) alone and in combination on cell growth relative to vehicle-treated control cells. Cells were grown in defined medium on MSS-DLL1 feeder cells. Cell counts were determined after 3 days of treatment.

Apoptosis assays. Caspase 3 and 7 activities were measured using the Caspase-Glo 3/7 assay system (Promega). Following the treatment of KOPT-K1 cells with DMSO, GSI, PPZ, or GSI plus PPZ, 5,000 cells from each sample were plated in the white-walled 96-well plates with a final volume of 100 \( \mu l \). An equal volume of Caspase-Glo 3/7 reagent was then added to each well. After 1 hour at room temperature, luminescence was measured with a SpectraMax M3 microplate reader (Molecular Devices). As a second measure of apoptosis, cells were stained with annexin V and PI using Apoptosis Detection Kit I (BD Biosciences) per the manufacturer’s instructions, and then analyzed in a FACSalibur flow cytometer (BD Biosciences) using Cell Quest software.
Marine xenograft assay. Retrovirus was made by transfecting MLCP, an MSCV-based retrovector encoding firefly luciferase, mCherry, and puro-mycin phosphotransferase, into Phoenix GP/293T cells (provided by David Williams, Boston Children’s Hospital). Transduced human hTALL2 T-ALL cells were isolated based on mCherry expression using a FACSAria II SORP UV cell sorter (BD Biosciences). Luciferized hTALL2 cells were injected intrascaffold into NOD/SCID II SORP UV cell sorter (BD Biosciences). Luciferized hTALL2 cells were subcutaneously injected and followed for tumor development using bioluminescence imaging as described (73). To assess the antileukemic activity of PPZ, mice were treated daily with 10 mg/kg of PPZ by intraperitoneal injection, and T-ALL growth was monitored by bioluminescence. To assess the effect of PPZ on phosphorylation of PP2A substrates in T-ALL cells in vivo, mice bearing bone scaffolds loaded with hTALL2 cells were treated with a single dose of PPZ (10 mg/kg). Each lane corresponds to a different animal. The blot stained for phospho-p70S6K and total p70S6K was prepared from a second gel that was loaded with the same samples and run in parallel.

**Figure 9**

PPZ inhibits growth and dephosphorylates PP2A targets in primary human T-ALL cells in vivo. (A) and (B) Effects of treatment with PPZ (10 mg/kg/d) on growth of human hTALL2 cells in NSG mice. (A) Effects on tumor cell growth were assessed by measuring the bioluminescence of luciferized hTALL2 cells in subcutaneous calcium phosphate scaffolds seeded with human mesenchymal stem cells. Arrow indicates the time of initiation of PPZ treatment. *P < 0.0182. (B) Spleen weights of treatment and control mice at times of necropsy. *P < 0.05. (C) Western blots prepared from hTALL2 cells harvested from engrafted mice 3 hours after a single dose of PPZ (10 mg/kg). Each lane corresponds to a different animal. The blot stained for phospho-p70S6K and total p70S6K was prepared from a second gel that was loaded with the same samples and run in parallel.


