DOT1L Inhibitor EPZ-5676 Displays Synergistic Antiproliferative Activity in Combination with Standard of Care Drugs and Hypomethylating Agents in MLL-Rearranged Leukemia Cells

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ABSTRACT

EPZ-5676 [(2R,3R,4S,5R)-2-[(6-amino-9H-purin-9-yl)-5-(((1R,3S)-3-(2-(5-(tert-buty-1H-benzo[d][1,2,5]imidazol-2-yl)ethyl)cyclobutyl)(isopropyl)amino)methyl]tetrahydrofuran-3,4-diol], a small-molecule inhibitor of the protein methyltransferase DOT1L, is currently under clinical investigation for acute leukemias bearing MLL-rearrangements (MLL-r). In this study, we evaluated EPZ-5676 in combination with standard of care (SOC) agents for acute leukemias as well as other chromatin-modifying drugs in cellular assays with three human acute leukemia cell lines: MOLM-13 (MLL-AF9), MV4-11 (MLL-AF4), and SKM-1 (non-MLL-r). Studies were performed to evaluate the antiproliferative effects of EPZ-5676 combinations in a cotreatment model in which the second agent was added simultaneously with EPZ-5676 at the beginning of the assay, or in a pretreatment model in which cells were incubated for several days in the presence of EPZ-5676 prior to the addition of the second agent. EPZ-5676 was found to act synergistically with the acute myeloid leukemia (AML) SOC agents cytarabine or daunorubicin in MOLM-13 and MV4-11 MLL-r cell lines. EPZ-5676 is selective for MLL-r cell lines as demonstrated by its lack of effect either alone or in combination in the nonrearranged SKM-1 cell line. In MLL-r cells, the combination benefit was observed even when EPZ-5676 was washed out prior to the addition of the chemotherapeutic agents, suggesting that EPZ-5676 sets up a durable, altered chromatin state that enhances the chemotherapeutic effects. Our evaluation of EPZ-5676 in conjunction with other chromatin-modifying drugs also revealed a consistent combination benefit, including synergy with DNA hypomethylating agents. These results indicate that EPZ-5676 is highly efficacious as a single agent and synergistically acts with other chemotherapeutics, including AML SOC drugs and DNA hypomethylating agents in MLL-r cells.

Introduction

Leukemias bearing recurrent translocations at the 11q23 locus are collectively referred to as MLL-rearranged (MLL-r) leukemia, and the occurrence of this genetic lesion is associated with a poor prognosis. Overall survival for 5 years for adult acute myeloid leukemia (AML) patients harboring balanced 11q23 translocations is between 10 and 20% (Byrd et al., 2002). Patients with this form of acute leukemia are currently treated by chemotherapy, most commonly using cytarabine (Ara-C) and daunorubicin, or by hematopoietic stem cell transplantation (Mrozek et al., 1997; Tamai and Inokuchi, 2010; Burnett et al., 2011). Either treatment modality is associated with a relatively poor response rate of approximately 45% among MLL-r patients (Mrozek et al., 1997; Byrd et al., 2002; Balgobind et al., 2009). Therefore, new treatment modalities for MLL-r have been of great interest to the clinical community.

Translocations at the 11q23 locus target the MLL gene and result in the expression of an oncogenic fusion protein comprising the amino-terminus of MLL fused in frame to one of over 70 potential fusion partners. The vast majority of such fusion partners are derived from the AF or ENL families of proteins (Hess, 2004; Krivtsov and Armstrong, 2007; Slany, 2009; Neff and Armstrong, 2013). The MLL protein is a transcriptional regulator with histone methyltransferase (HMT) activity specific for histone H3 at lysine 4 (H3K4) (Milne et al., 2002; Nakamura et al., 2002; Chang et al., 2010). In the context of the MLL-r–associated fusion proteins, MLL loses its catalytic domain. However, a unifying feature of many of the common MLL fusion partner proteins is the ability to bind to another HMT known as DOT1L (Okada et al., 2007; Bitoun et al., 2007; Mueller et al., 2007; Mohan et al., 2010; Park et al., 2013). Studies using DOT1L inhibitors have demonstrated a consistent combination benefit with MLL-r cell lines. EPZ-5676 is selective for MLL-r cell lines as demonstrated by its lack of effect either alone or in combination in the nonrearranged SKM-1 cell line. In MLL-r cells, the combination benefit was observed even when EPZ-5676 was washed out prior to the addition of the chemotherapeutic agents, suggesting that EPZ-5676 sets up a durable, altered chromatin state that enhances the chemotherapeutic effects. Our evaluation of EPZ-5676 in conjunction with other chromatin-modifying drugs also revealed a consistent combination benefit, including synergy with DNA hypomethylating agents. These results indicate that EPZ-5676 is highly efficacious as a single agent and synergistically acts with other chemotherapeutics, including AML SOC drugs and DNA hypomethylating agents in MLL-r cells.
11q23 chromosomal rearrangement. synergy with other agents in leukemia cells that do not bear an azacitidine and decitabine. Remarkably, this synergy is obtained synergistically with the AML SOC chemotherapeutic agents approved DNA methyltransferase (DNMT) inhibitors. Here, we report the results of these studies. We find that EPZ-5676 acts (SOC) treatments for AML, as well as in combination with another class of epigenetic-targeted drugs, such as the currently approved DNA methyltransferase inhibitor. Here, we report the results of these studies. We find that EPZ-5676 acts synergistically with the AML SOC chemotherapeutic agents Ara-C and daunorubicin as well as with the DNMT inhibitors azacitidine and decitidine. Remarkably, this synergy is observed not only when the EPZ-5676 and the second drug are coadministered to cells, but also when cells are pretreated with EPZ-5676, this compound is washed out, and then the second drug is applied to the cells. Consistent with the hypothesized mechanism of action of EPZ-5676, we did not observe single-agent activity for this compound, nor did we observe any synergy with other agents in leukemia cells that do not bear an 11q23 chromosomal rearrangement.

Materials and Methods

Cell Lines. The biphenotypic leukemia cell line MV4-11 (MLL-AF4) (CRL-9591) was obtained from American Type Culture Collection (Manassas, VA). AML cell lines MOLM-13 (MLL-AFB) (ACC 554) and SKM-1 (ACC 547) were obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). MV4-11 cells were maintained in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal bovine serum. MOLM-13 and SKM-1 cells were maintained in RPMI medium supplemented with 10% fetal bovine serum. They were cultured in flasks or plates in a humidified 37°C, 5% CO2 atmosphere.

Compounds. EPZ-5676 was synthesized by Epizyme (Cambridge, MA). Ara-C, vincristine, and prednisolone were purchased from SelleckChem (Houston, TX). Mitoxantrone, daunorubicin, and azacitidine were purchased from Sigma-Aldrich (St. Louis, MO). LSD1 inhibitor II ((trans)-2-(3,5-difluoro-2-phe nethylenepheryl) cyclopropanamide) and tranilupromine were purchased from EMD Millipore (Billerica, MA). Mafosfamide was purchased from Santa Cruz Biotechnology (Dallas, TX). JQ1 ([6S]-4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-$\beta$][1,2,4]triazolo[4,3-$\alpha$]-1,4)diazepine-6-acetic acid, 1,1-dimethyl ethyl ester) was purchased from Cayman Chemical (Ann Arbor, MI). IBET-151 [(7-[[5,5-dimethyl-4-isoxazolyl]-1,3-dihydroxy-8-methoxy-1-{[1R]-1-pyridinyl}ethyl]-2H-imidazo[4,5-c]quinolin-2-one hydrochloride] was purchased from Tocris Biosciences (Bristol, UK).

Proliferation Assays. Proliferation studies were performed using MOLM-13, MV4-11, and SKM-1 cell lines in vitro to evaluate both the combinatorial effect of compounds on cell killing and the durability of the effect by washing out one of the agents. For all assays described, the cell counts were measured by ATP quantitation using the CellTiter-Glo reagent from Promega (Madison, WI), and luminescence values corresponded to the amount of ATP in a given well.

SOC drugs were tested in combination with EPZ-5676 to study their effect on cell proliferation in a pretreatment model, with or without washout, or in a cotreatment model. The cotreatment model was used to further investigate the combinatorial effect of EPZ-5676 with other agents of interest.

Prior to performing the combination experiments, the IC50 value of each compound was determined in each cell line, which served to appropriately bracket the concentration ranges around this half-maximal inhibition value in the combination matrix.

In the pretreatment model, exponentially growing cells were seeded in flasks (5 × 104 cells/ml for MV4-11 and 3 × 104 cells/ml for MOLM-13) and pretreated with seven concentrations of EPZ-5676 or gemcitabine (DMSO) for several days, followed by cotreatment with EPZ-5676 and SOC. MV4-11 assays were a 4 + 3 model and MOLM-13 assays were a 7 + 3 model. Pretreatment concentrations of EPZ-5676 were tested by performing a series of 2-fold dilutions of EPZ-5676 with a top concentration of 500 and 650 nM for MV4-11 and MOLM-13 cells, respectively. On days 4 (MV4-11) or 7 (MOLM-13), cell densities were normalized to 5 × 10^4 cells/ml, and cells were then plated to a 96-well plate containing EPZ-5676 with the second agent in a matrix format for the 3-day cotreatment phase. Assay-ready plates were prepared with the HP-D300 digital compound dispenser (Tecan, Männedorf, Switzerland). The combinatorial matrix allowed for several constant ratios to be interrogated simultaneously. The constant ratio was calculated by normalizing the concentration of the second test compound to EPZ-5676, e.g., EPZ-5676 at 50 nM to the second combination agent at 100 nM was calculated as a 1:2 constant ratio. The concentration matrix was designed to evaluate the single-agent activity by treating cells with one agent paired with DMSO, which was normalized to 0.1% v/v across the plate. When washout experiments were performed, EPZ-5676 was excluded in the 3-day incubation after pretreatment.

The cotreatment model was established to understand the effect of simultaneous dosing of both agents beginning at time 0 and ending on day 7. In the cotreatment model, exponentially growing MV4-11, MOLM-13, or SKM-1 cells were seeded in 96-well plates at 1.25 × 10^4, 3 × 10^4, and 6 × 10^4 cells/ml, respectively, and treated for 7 days with the combination of EPZ-5676 and a compound of interest in a matrix format as previously described. The top concentration of EPZ-5676 tested was 50, 250, and 4000 nM for MV4-11, MOLM-13, and SKM-1, respectively, and compounds were preloaded in DMSO in a 2-fold dilution series for a total of eight concentrations tested.

A third model was established to study the effect of addition of Ara-C by measuring the 10-day proliferation of cells pretreated with Ara-C in a 3 + 7 model. This experiment was performed by first pretreating MOLM-13 cells with increasing concentrations of Ara-C for 3 days (concentration range 1.9–250 nM). Ara-C was then washed out, and the cell numbers were normalized and either treated with EPZ-5676 alone or cotreated with EPZ-5676 and Ara-C in a matrix format for an additional 7 days. For all experimental models, maximum and minimum inhibition (DMSO alone) controls were used in each plate to calculate the
fractional effect (Fa) of a test well. DMSO concentration was kept at 0.1% v/v. The drug combination analysis was performed as described below in "Data Analysis."

Cell Treatment of Analysis of Mechanism of Cell Death Studies. To understand the mechanism of cell death, MOLM-13 cells were seeded in flasks at 3 × 10⁵ cells/ml and pretreated with EPZ-5676 (625–10 nM, 2-fold serial dilutions) for 7 days. On day 7, cells were split and normalized to a seeding density of 5 × 10⁵ cells/ml. Cells were then treated with EPZ-5676 (78 and 156 nM) alone, in combination with Ara-C (31 and 63 nM), or in combination with daunorubicin (7.5 and 15 nM) to induce a synergistic antiproliferative response. Again, on day 10, cells were normalized and redosed with individual compound or a combination of EPZ-5676 and SOC. Samples of cells were taken at days 7, 10, and 14.

Flow Cytometric Analysis of Cell Cycle and Annexin V. Flow cytometric analysis was performed to evaluate the fraction of cells in each phase of the cell cycle and to determine cell death by apoptosis. Cells were treated (as described previously in "Cell Treatment of Analysis of Mechanism of Cell Death") alone or in combination with EPZ-5676 and SOC and harvested on days 7, 10, and 14, and samples were divided to allow simultaneous analysis of cell cycle and annexin V staining. Apoptosis was determined using the Guava Nexa Assay (Millipore) and cells were prepared according to the manufacturer's recommendations. Samples were analyzed using the Guava EasyCyte Plus System (Millipore). For cell cycle analysis, cells were pelleted by centrifugation at 200g for 5 minutes at 4°C, washed twice with ice-cold phosphate-buffered saline (PBS), then fixed with 70% ice-cold ethanol. Following fixation, cells were washed with PBS and stained with the Guava cell cycle reagent (Millipore) for 30 minutes. Samples were analyzed using the Guava EasyCyte Plus System.

Analysis of CD11b and CD14 Expression by Flow Cytometry. MOLM-13 cells were treated in the presence of EPZ-5676, Ara-C, and daunorubicin as single agents or in combination of EPZ-5676 and Ara-C or daunorubicin (as described previously in "Cell Treatment of Analysis of Mechanism of Cell Death"). On days 7, 10, and 14, cells were collected for analysis. Samples were washed twice with PBS followed by fixation in 4% formaldehyde for 10 minutes at 37°C. After fixation, cells were washed and blocked with blocking buffer (0.5% bovine serum albumin in PBS) for 10 minutes at room temperature. Cells (5 × 10⁶) were then incubated in the presence of mouse anti-CD14 antibody, mouse anti-CD11b antibody, or mouse anti-IgG isotype control, all fluorescein isothiocyanate–labeled (Millipore), for 1 hour at room temperature while rotating. Cells were washed, resuspended in PBS, and 5000 events were analyzed using ExpressPro software on the Guava EasyCyte Plus System.

Quantitative Polymerase Chain Reaction. MOLM-13 cells were treated with DMSO, 78 nM, or 40 nM of EPZ-5676 for 7 days. Cells were split to 50,000 cells/ml and re-treated with DMSO, 78 nM, or 40 nM of EPZ-5676 alone or in combination with 78 nM EPZ-5676 + 31 nM Ara-C or 40 nM EPZ-5676 + 3.8 nM daunorubicin for 3 additional days. Cells were harvested and total mRNA was extracted from cell pellets using the RNeasy Plus Mini Kit (Qiagen; Germantown, MD). cDNA was made using the High Capacity cDNA Reverse Transcriptase Kit (4368813; Invitrogen, Carlsbad, CA). TaqMan probe-based quantitative polymerase chain reaction was carried out with the ViiA 7 Real-Time PCR Systems (Applied Biosystems [AB], Foster City, CA) using TaqMan Fast Advanced Master Mix (4444964; AB) and TaqMan primer/probe sets for β-actin, CD11b, and CD14 (4333762F, Hs00355885_m1, Hs02621496_s1; Invitrogen). Gene expression was normalized to housekeeping gene RPLP0 (4333761F; AB) and fold change compared with DMSO was calculated using the ΔΔct method.

Data Analysis. The drug combination analysis was performed using the Chou-Talalay method (Chou, 2006). Synergy quantification is performed using the CalcuSyn for Windows version 2.1 software (Biosoft, Cambridge, UK). The combination index (CI) equation offers a quantitative definition for additivity (CI = 1), synergism (CI < 1), and antagonism (CI > 1). This equation uses Fa values from a constant ratio of drug combination to determine CI values. The resulting plot (Pa-CI) shows the resultant CI values bracketed by 95% confidence intervals. CI values <1 with confidence interval lines also below 1 indicate statistically significant synergism. For combination studies in the MOLM-13 and MV4-11 cell lines, the 50% inhibitory concentrations of the single agents were referred to as D₅₀ (rather than IC₅₀).

Additionally, synergism and antagonism were described based on the CI values according to the Chou and Talalay guidelines (CI values: 0–0.1, very strong synergism; 0.1–0.3, strong synergism; 0.3–0.7, synergism; 0.7–0.85, moderate synergism; 0.85–0.9, slight synergism; 0.9–1.1, nearly additive; 1.10–1.20, slight antagonism; 1.20–1.45, moderate antagonism; 1.45–3.3, antagonism; 3.3–10, strong antagonism; 10, very strong antagonism).

For combinations of EPZ-5676 and a test compound in the SKM-1 cell line, where only the latter compound showed more than 50% inhibition as a single agent, dose responses were plotted using GraphPad Prism (GraphPad, La Jolla, CA), and 50% inhibitory concentrations were interpolated from the four-parameter logistic model. The quantitation of the antiproliferative effect between the two compounds was calculated as fold potency shift of the test compound in the presence of several concentrations of EPZ-5676. Combination benefit or enhancement of the antiproliferative activity was demonstrated when the presence of EPZ-5676 produced a leftward shift of the IC₅₀. In contrast, antagonism was demonstrated by a rightward shift of the IC₅₀. This shift was considered significant when confidence intervals for the calculated IC₅₀ values in the presence of EPZ-5676 did not overlap with those of the IC₅₀ value of the test compound alone. For experiments in the SKM-1 cell line with combinations of EPZ-5676 with Ara-C, daunorubicin, or azacitidine, the 1/α constant was calculated to quantitate further the combinatorial effect (methods for calculation of α and its reciprocal value are described in the Supplemental Material).

Results

EPZ-5676 Induces a Synergistic and Durable Anti-proliferative Effect in Combination with AML Standard of Care Drugs. We evaluated the effect of EPZ-5676 on the proliferation of the MLL-r leukemia cell lines MOLM-13 (AML) and MV4-11 (biphenotypic leukemia) when used in combination with the SOC drugs for AML, Ara-C and daunorubicin. We have previously shown that the antiproliferative effects of EPZ-5676 on cultured cells require several days to manifest (Daigle et al., 2013). Proliferation assays were, therefore, performed over a period of 7–10 days. We have previously demonstrated that EPZ-5676 treatment of MV4-11 cells decreased histone H3 lysine 79 methylation and expression of key MLL fusion target genes, such as HOX9 (Daigle et al., 2013). We confirmed that this was also the case in MOLM-13 cells (Supplemental Fig. 4). To evaluate combination effects, cells were treated according to the pretreatment model described in Materials and Methods (i.e., treatment with EPZ-5676 alone for 4–7 days followed by cotreatment with EPZ-5676 plus SOC agent for 3 days). Synergistic antiproliferative activity was observed with both Ara-C and daunorubicin under this regimen (Fig. 1). The synergistic antiproliferative activity of EPZ-5676 in combination with AML SOC agents was also observed when cells were treated according to the cotreatment model described in Materials and Methods (i.e., treatment with EPZ-5676 plus SOC agent for 7 days; see Table 1). Intriguingly, synergistic antiproliferative activity, albeit somewhat reduced when compared with cotreatment, was observed in MOLM-13 and MV4-11 MLL-r cells even when EPZ-5676 was removed (i.e., washed out) prior to the addition of the SOC agent (Fig. 2). These data are remarkable in that they imply a durable reprogramming of the epigenetic status of these cells by EPZ-5676.
that renders them more acutely sensitive to chemotherapeutic agents, even when the DOT1L inhibitor has been removed from the cellular environment. This result is consistent with the kinetics of the EPZ-5676 effect on histone methylation at the DOT1L substrate site, H3K79. In previous studies, we have shown that 4 days of treatment with EPZ-5676 is sufficient to deplete cellular levels of H3K79me2 by \( \approx 80\% \) (Daigle et al., 2013). H3K79 methylation remained depleted for 3 days following subsequent removal of EPZ-5676 by washout. After this 3-day latency period, the level of H3K79me2 slowly returned to pretreatment levels over the course of an additional 4 days. Hence, treatment of \( MLL-r \) cells with EPZ-5676 results in durable inhibition of H3K79 methylation, which in turn results in sensitization of these cells to chemotherapy-induced cell killing.

To further test the flexibility of dosing schedules that might afford synergistic cell killing, we pretreated MOLM-13 cells with the chemotherapeutic agent Ara-C for 3 days, washed out this drug, and then treated the cells with EPZ-5676 for an additional 7 days. As illustrated in Fig. 3, this sequential treatment schedule resulted in essentially the same level of synergistic cell killing as seen when both drugs were coadministered to cells simultaneously. These results offer the possibility of a highly flexible dosing schedule for combinations of EPZ-5676 and chemotherapies. Whereas both single-agent activity and strong synergy with Ara-C and daunorubicin were seen for EPZ-5676 in the \( MLL-r \) cell lines MV4-11 and MOLM-13, no effect of EPZ-5676 was observed in the non-\( MLL-r \) AML cell line SKM-1. EPZ-5676 showed no single-agent activity in SKM-1 cells, nor did it affect the antiproliferative activity of either chemotherapeutic agent in this cell line (Supplemental Fig. 1). The lack of activity of EPZ-5676 in SKM-1 cells is completely consistent with the proposed mechanism of action of this drug. In previous studies, we have demonstrated that, although EPZ-5676 and related compounds also inhibit intracellular DOT1L activity—as evidenced by concentration-dependent inhibition of H3K79 methylation—in non-\( MLL-r \) leukemia cell lines, this enzyme inhibition only translates into an antiproliferative effect for those leukemia cells bearing an 11q23 chromosomal translocation (Daigle et al., 2011, 2013).

**EPZ-5676 Increases Expression of Differentiation Markers and Apoptosis as Single Agent and in Combination with AML Standard of Care Drugs.** We wished to describe the phenotypic effects underlying synergistic antiproliferative activity observed with EPZ-5676 and AML SOC agent combinations in more detail. EPZ-5676 and related compounds have previously been reported to promote apoptosis and differentiation in \( MLL-r \) cells. Consistent with this, EPZ-5676 alone...
TABLE 1
Summary of combination studies of EPZ-5676 with AML and ALL standard of care drugs and chromatin-modifying agents

<table>
<thead>
<tr>
<th>Rationale</th>
<th>Compound</th>
<th>MOLM-13 (MLL-AF9)</th>
<th>MV4-11 (MLL-AF4)</th>
<th>SKM-1 (Non-MLL-r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML standard of care</td>
<td>Ara-C</td>
<td>Strong synergy</td>
<td>Strong synergy</td>
<td>No effect*</td>
</tr>
<tr>
<td>DNA methyltransferase inhibitors</td>
<td>Daunorubicin</td>
<td>Synergy</td>
<td>Strong synergy</td>
<td>No effect*</td>
</tr>
<tr>
<td>Histone deacetylase inhibitors</td>
<td>Azacitidine</td>
<td>Synergy</td>
<td>Synergy</td>
<td>No effect*</td>
</tr>
<tr>
<td>Histone deacetylase inhibitors</td>
<td>Decitabine</td>
<td>Synergy</td>
<td>Synergy</td>
<td>No effect*</td>
</tr>
<tr>
<td>Demethylase inhibitors</td>
<td>Vorinostat</td>
<td>Additive/synergy</td>
<td>Antagonistic</td>
<td>N/D</td>
</tr>
<tr>
<td>Demethylase inhibitors</td>
<td>Panobinostat</td>
<td>Synergy</td>
<td>Antagonistic</td>
<td>N/D</td>
</tr>
<tr>
<td>Bromodomain inhibitors</td>
<td>IBET-151</td>
<td>Synergy</td>
<td>Strong synergy</td>
<td>No effect*</td>
</tr>
<tr>
<td>ALL standard of care</td>
<td>JQ-1</td>
<td>Additive</td>
<td>Additive</td>
<td>No effect*</td>
</tr>
<tr>
<td></td>
<td>Mitoxantrone</td>
<td>Synergy</td>
<td>Synergy</td>
<td>No effect*</td>
</tr>
<tr>
<td></td>
<td>Methotrexate</td>
<td>Additive</td>
<td>Additive</td>
<td>No effect*</td>
</tr>
<tr>
<td></td>
<td>Mafosfamide</td>
<td>Strong synergy</td>
<td>Strong synergy</td>
<td>No effect*</td>
</tr>
<tr>
<td></td>
<td>Prednisolone</td>
<td>Antagonisticb</td>
<td>Antagonisticb</td>
<td>Enhancementbd</td>
</tr>
<tr>
<td></td>
<td>Vincristine</td>
<td>Additive</td>
<td>Additive</td>
<td>No effect*</td>
</tr>
</tbody>
</table>

IBET-151, 7-(3,5-dimethyl-4-isoxazolyl)-1,3-dihydroxy-8-methoxy-1-[(1R)-1-(2-pyridinyl)ethyl]-2Himidazo[4,5-c]quinolin-2-one hydrochloride; N/D, not determined.

*No enhancement was observed based on analysis of the IC<sub>50</sub> shift of the test compound in the presence of EPZ-5676. Analysis of IC<sub>50</sub> shifts is described under Materials and Methods.

IC<sub>50</sub> of test compound not achieved.

Methotrexate showed antagonistic effect in combination with EPZ-5676 at some constant ratios.

Enhancement or shift in IC<sub>50</sub> was observed at concentrations of EPZ-5676 of 2000 nM and above.

induces a concentration-dependent increase in apoptotic cells (as measured by annexin V staining) after 7 days of treatment of MOLM-13 cells (Fig. 4A). The total content of viable cells decreases with EPZ-5676 concentration according to a classic Langmuir isotherm, with a midpoint value (EC<sub>50</sub>) of 364 ± 18 nM, and this trend is exactly mirrored by the increasing content of apoptotic cells (sum of early and late-stage apoptosis). We next evaluated the kinetics for induction of apoptosis at fixed time points over a 14-day course of treatment of MOLM-13 cells treated with DMSO (as a control), 156 nM EPZ-5676, 63 nM Ara-C, or a combination of EPZ-5676 and Ara-C (at the same concentrations as for the single-agent treatments). Ara-C by itself induced a modest increase in apoptotic cell population over the cotreatment period, whereas EPZ-5676 treatment led to a much more robust induction of apoptosis over the same time course. The combination of the two drugs led to enhanced apoptosis in MOLM-13 cells (Fig. 4B). Apoptotic cell content was also assessed by measuring the percentage of cells in the sub-G1 phase of the cell cycle. The data for the sub-G1 cell population are graphed as a kinetic plot in Fig. 4C. This plot makes clear that Ara-C treatment alone has minimal effect on the sub-G1 population of MOLM-13 cells over the 7-day treatment course, whereas treatment with EPZ-5676 leads to a moderate, time-dependent increase in sub-G1 population. When EPZ-5676 and Ara-C are combined, a significant increase in the population of sub-G1 cells at 10 and 14 days is realized with a concomitant increase in the rate of sub-G1 population growth as well. Figure 4, D–F, illustrates the distribution of the G1, S, and G2/M cell cycle stages, respectively, at various time points for MOLM-13 cells treated with DMSO (control), 156 nM EPZ-5676, 63 nM Ara-C, or a combination of EPZ-5676 and Ara-C. Similar results were observed when EPZ-5676 was combined with daunorubicin (data not shown).

In addition to driving apoptotic cell death, EPZ-5676 and Ara-C either as single agents or in combination promote time- and concentration-dependent upregulation of the differentiation markers CD11b and CD14 (Fig. 5) in MLL-r MOLM-13 cells. The same effect was observed by day 10 with daunorubicin as a single agent and in combination with EPZ-5676 (Supplemental Fig. 2). In all cases, the degree of differentiation marker upregulation was greater with the combination of agents than with either agent alone. This significant upregulation was also shown by gene expression analysis of differentiation markers in MOLM-13 cells treated alone or in combination with EPZ-5676 and either Ara-C or daunorubicin (Supplemental Fig. 5). Taken together, these results demonstrate that the synergistic antiproliferative activity observed by combining EPZ-5676 with AML SOC agents is due to an enhanced ability of drug combinations over single agents to induce apoptosis and differentiation in MLL-r cells.

EPZ-5676 Demonstrates Combination Benefit with Acute Lymphoblastic Leukemia Standard of Care Drugs. MLL-r is also found in acute lymphoblastic leukemia (ALL) and is primarily associated with infants (children younger than 12 months). This subset of ALL has a poor prognosis when compared with the ALL patients without the 11q23 translocation. Long-term event-free survival in infants harboring MLL-r has been reported to be between 28 and 45%. These rates are much lower than non-MLL-r patients who have survival rates approaching 90% (Pieters et al., 2007; Bhajwani et al., 2009; Inaba et al., 2013). Similar to the AML SOC, we wanted to evaluate combination of EPZ-5676 with current ALL therapies that include mitoxantrone, methotrexate, mafosfamide, prednisolone, and vincristine (Pieters et al., 2007; Inaba et al., 2013). The results of these combinations are summarized in Table 1 (see also Supplemental Table 1). We observed synergism or additive effects with all of the ALL SOC agents in combination with EPZ-5676 with the exception of prednisolone, where antagonism was observed in MLL-r cell lines. No enhancement of the antiproliferative single-agent activity of ALL SOC drugs was seen when combined with EPZ-5676 in the non-MLL-r cell line SKM-1 with the exception of prednisolone, where enhanced antiproliferative activity was observed in the presence of EPZ-5676 concentrations greater than 1000 nM.
The basis for this enhancement in prednisolone activity is unknown; however, we note that these EPZ-5676 concentrations are much higher than those required for maximal efficacy in preclinical models.

**EPZ-5676 Demonstrates Strong Synergy with DNMT Inhibitors and Chromatin-Modifying Agents in MLL-Rearranged Cell Lines.** There is considerable evidence that epigenetic regulation of gene transcription results from the combinatorial effects of distinct covalent modifications of chromatin components, including histone methylation, histone acetylation, other covalent histone modifications, and direct methylation of chromosomal DNA at CpG islands by the DNMTs (Kouzarides, 2007; Arrowsmith et al., 2012). With this in mind, we tested the impact of combining the HMT inhibitor EPZ-5676 with compounds that affect their pharmacology by inhibition of other chromatin regulators, such as histone deacetylases (HDACs), histone demethylases, acetyl-lysine reader domains (bromo-domains), and DNMTs. The results of these combinations are summarized in Table 1 (see also Supplemental Table 1). Most of the conditions tested demonstrated synergy or additivity between EPZ-5676 and these compounds, but there were also examples of antagonism, including HDAC inhibitors in the context of MOLM-13 cells. The DNMT inhibitors decitabine and azacitidine demonstrated synergistic antiproliferative activity in MLL-r cells when combined with EPZ-5676. In contrast, and again consistent with the mechanism of action of EPZ-5676, this compound had no impact on the antiproliferative activity of either DNMT inhibitor when tested in the non–MLL-r leukemia cell line SKM-1 (Table 1). Figure 6 illustrates representative data for the strong synergistic effects of combining azacitidine and EPZ-5676 in the MV4-11 and MOLM-13 cell lines. No potency enhancement was seen when SKM-1 cells were treated with a combination of EPZ-5676 and azacitidine (Supplemental Fig. 3).

**Discussion**

Advances in genomic understandings of human cancers have provided a rational basis for the identification of oncogenic, driver alterations in specific cancer types. Surveys of these oncogenic lesions have led to a broad understanding of the molecular underpinnings of cancer, in terms of both metabolic and signaling pathways that are commonly co-opted in disease (Hanahan and Weinberg, 2011), as well as specific molecular targets that are genetically altered in a manner that causes or facilitates the hyperproliferative phenotype of cancer. Thus, today a significant portion of basic cancer research and applied drug discovery research is focused on the identification and validation of specific molecular targets for therapeutic intervention in cancer (Patel et al., 2012; Jabbour Fig. 2. Synergistic antiproliferative activity between EPZ-5676 and AML SOC agents is maintained following EPZ-5676 washout prior to the addition of the SOC agents in MLL-r leukemia cell lines. MOLM-13 and MV4-11 MLL-r cells were treated according to the pretreatment model described in Materials and Methods (with EZP-5676 washout). EPZ-5676 concentrations spanned a range known to be efficacious in preclinical models. Data are representative of two biologic experiments. The gray oval on the Fa-CI plots indicates a region of synergy. (A) Fa-CI plot shows synergistic combination of EPZ-5676 and Ara-C at a 20:1 constant ratio in MOLM13 cells (data plotted represent a concentration range of 39.5–312.5 nM for EPZ-5676 and 1.95–15.6 nM for daunorubicin). (B) Fa-CI plot shows synergistic combination of EPZ-5676 and daunorubicin at a 1:8 constant ratio in MV4-11 cells (data plotted represent a concentration range of 15.6–500 nM for EPZ-5676 and 0.78–12.5 nM for daunorubicin). For reference, in MOLM-13 cells, the \( D_{50} \) for EPZ-5676 is 0.21 \( \mu \text{M} \), the \( D_{50} \) for Ara-C is 0.053 \( \mu \text{M} \), and the \( D_{50} \) for daunorubicin is 0.0040 \( \mu \text{M} \). In MV4-11 cells, the \( D_{50} \) for EPZ-5676 is 0.16 \( \mu \text{M} \), the \( D_{50} \) for Ara-C is 0.33 \( \mu \text{M} \), and the \( D_{50} \) for daunorubicin is 0.0022 \( \mu \text{M} \).
pretreated with several doses of Ara-C (2-fold serial dilutions, 1.9 M) washout of Ara-C prior to the addition of EPZ-5676. MOLM-13 cells were pretreated with Ara-C prior to cotreatment with EPZ-5676, and the effect is durable upon removal from the cultures prior to treatment with EPZ-5676. Concentrations of Ara-C in excess of 31.25 nM produced cell death and therefore could not be included in the calculation of combination index. For reference, the DM_{10} for EPZ-5676 for the 7-day cotreatment phase as a single agent was 0.052 μM, and the DM_{10} for Ara-C in the 10-day assay was 0.017 μM (no Ara-C washout) and 0.036 μM (Ara-C washout).

Despite this paradigm shift in cancer treatment, clinical oncology today continues to rely heavily on more traditional chemotherapeutic approaches. Although these traditional chemotherapies are less targeted, and therefore prone to greater safety concerns, they have nevertheless demonstrated broad activity in the treatment of cancer patients. Most commonly, cancer therapies of varying modality are combined in clinical use to offer the best compromise between potent, anticancer efficacy and patient safety and comfort. Hence, it is imperative that as new, targeted therapies enter the clinic, the research community understands how these new therapeutic modalities may combine with existing drugs that are in current clinical use for a specific cancer indication. The goals of this type of research are 2-fold. First, it is critical that one understands any antagonistic relationship between a new therapeutic agent and current SOC therapies that might contraindicate the coadministration of the two. Second, preclinical and clinical studies of drug combinations may reveal a greater-than-additive effect of the combined drugs that would provide significant benefit to patients; drugs that, when combined, produce greater efficacy than would be expected from summing of their individual activities are said to behave synergistically.

EPZ-5676 is a highly selective and potent inhibitor of the protein methyltransferase enzyme DOT1L, and is being pursued as a targeted cancer therapeutic for patients with 11q23 chromosomal translocations as the driver alteration in MLL-r leukemia. This compound has demonstrated potent, single-agent activity as a selective cytotoxic for MLL-r leukemia cells with minimal impact on non–MLL-r cells in preclinical models, and has recently begun phase 1 clinical testing (Daigle et al., 2013). AML patients, including those bearing MLL-rearrangements, are most commonly treated today with the chemotherapies Ara-C and daunorubicin, or by hematopoietic stem cell transplantation (Mrozek et al., 1997; Tamai and Inokuchi, 2010; Burnett et al., 2011). Our goal in the current study was, therefore, to determine the impact of combining EPZ-5676 with these SOC chemotherapeutics on the proliferation of MLL-r leukemia cells in culture.

As illustrated in Fig. 1 and Table 1, the combination of EPZ-5676 with either Ara-C or daunorubicin resulted in strongly synergistic antiproliferative activity when tested against the MLL-r leukemia cell lines MV4-11 and MOLM-13. These cell lines are derived from AML and acute biphenotypic leukemia, respectively, and express the two most commonly found MLL fusion proteins. MV4-11 cells bear a t(4;11)(q21;q23) translocation in which the MLL fusion partner protein is AF4, whereas MOLM-13 cells bear a t(9;11)(q23;p22) translocation in which the MLL fusion partner protein is AF9. In stark contrast to the strong synergy seen between EPZ-5676 and these chemotherapies in cells harboring 11q23 chromosomal translocations, no effect of EPZ-5676 was observed in the non–MLL-r leukemia cell line SKM-1, either as a single agent or in combination. This lack of activity in non–MLL-r leukemia is completely consistent with the proposed mechanism of action of EPZ-5676 and with previous studies of the drug as a single agent in a broad spectrum of leukemia cell lines (Daigle et al., 2013).

A universal characteristic of acute leukemias, such as MLL-r, is the accumulation of highly proliferative myeloblasts or lymphoblasts that have lost their ability to differentiate fully into mature leukocytes. In previous studies, we have demonstrated that EPZ-5676 and related compounds effect a reprogramming of chromatin modification and downstream transcriptomic activities that result in MLL-r leukemia cells starting down a path of differentiation/maturaton and apoptotic cell death (Daigle et al., 2011, 2013). Differentiation/maturaton can be characterized and quantified by cytometric assessment of cell surface markers of cell state, such as CD11b and CD14, two markers of leukocyte differentiation. In combination with Ara-C, both apoptotic (Fig. 4) and differentiation/maturation responses to EPZ-5676 treatment were augmented on day 10 with the greatest expression measured on day 14 (Fig. 5). Differentiation/maturation responses measured on day 10 show an increase in the expression of CD11b and CD14 when cells were cotreated with EPZ-5676 and daunorubicin, compared with daunorubicin alone. In combination with Ara-C or daunorubicin, both the apoptotic and differentiation/maturation responses to EPZ-5676 and AML leukemia cells showed an increase in the expression of CD11b and CD14 when cells were cotreated with EPZ-5676 and daunorubicin, compared with daunorubicin alone. In combination with Ara-C or daunorubicin, both the apoptotic and differentiation/maturation
responses to EPZ-5676 treatment were augmented (Fig. 5; Supplemental Fig. 2). Induction of blast differentiation and/or apoptosis is an established approach to resolution of human leukemias (Bruserud et al., 2000; Nowak et al., 2009). The ability of EPZ-5676 to cause blast differentiation and apoptosis by itself, and the enhancement of these effects by Ara-C and daunorubicin, bodes well for the effective use of this compound alone or with chemotherapy as a clinical treatment for MLL-r patients. It is noteworthy that the antileukemic effect of EPZ-5676 in combination with chemotherapy was tolerant of variation in dose schedule. In particular, the ability to pretreat cells with EPZ-5676, remove this compound by washout, and then dose the chemotherapeutic with retention of synergistic effect offers considerable flexibility in the clinical utilization of such combinations. This potential dosing schedule flexibility may prove quite useful not only in optimizing the efficacy of the combination therapy but also in mitigation of any chemotherapy-associated safety issues.

MLL-rarrangements are also found in a subset of ALL patients. We therefore tested the effect of combining EPZ-5676 with current ALL therapies that include mitoxantrone, methotrexate, mafosfamide, prednisolone, and vincristine (Pieters et al., 2007; Inaba et al., 2013). With the exception of prednisolone, all of these SOC agents demonstrated synergy or additivity with EPZ-5676, thereby providing a rationale for combining EPZ-5676 with current ALL SOC drugs for the treatment of MLL-r patients suffering from ALL.

Beyond current acute leukemia SOC chemotherapies, we also wished to explore the possibility of synergy between EPZ-5676, a HMT inhibitor, and other epigenetic-targeted compounds for treatment of MLL-r leukemias. Preclinical small-molecule inhibitor and genetic knockdown studies have provided some
Fig. 5. EPZ-5676 and Ara-C as single agents and in combination promote time- and concentration-dependent upregulation of the differentiation markers CD11b and CD14 in MLL-rearranged leukemia cells. MOLM-13 cells were treated as described under Materials and Methods for mechanism of cell death studies. EPZ-5676 and Ara-C as single agents and in combination promote time- and concentration-dependent upregulation of the differentiation markers CD11b and CD14. Histograms in black represent data from naïve cells. Red histograms represent data from DMSO-treated cells. Histograms in magenta and green represent the indicated concentrations of single agent or combination of the two agents. Results are representative of two biologic experiments. (A) Flow cytometry analysis for cell surface expression of CD11b shows time- and dose-dependent upregulation of the marker in cells treated with EPZ-5676 and Ara-C as single agents and in combination. (B) Flow cytometry analysis for cell surface expression of CD14 shows time- and dose-dependent upregulation of the marker in cells treated with EPZ-5676 and Ara-C as single agents and in combination. (C) Flow cytometry analysis for cell surface expression of IgG isotype control. Similar results were obtained in two independent experiments.
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The data further suggest that combinations of EPZ-5676 and DNMT inhibitors may also provide additional benefit to patients with MLL-r leukemia. These preclinical data provide a basis for rational hypothesis generation that must ultimately be tested in human clinical trials.

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References


Fig. 6. EPZ-5676 and azacitidine synergistically induce an antiproliferative effect in MLL-rearranged leukemia cells. MOLM-13 and MV4-11 cells were treated with EPZ-5676 and azacitidine as single agents and in combination according to the protocol model described under Materials and Methods. The gray oval on the Fa-CI plots indicates a region of synergy. (A) Fa-CI plot shows synergistic antiproliferative activity when MOLM-13 cells were cotreated with EPZ-5676 and azacitidine (data plotted represent a concentration range of 39.5–625 nM for EPZ-5676 and 156–2500 nM for azacitidine). (B) Fa-CI plot shows synergistic antiproliferative activity when MV4-11 cells were cotreated with EPZ-5676 and azacitidine (data plotted represent a concentration range of 1.9–125 nM for EPZ-5676 and 19.5–1250 nM for azacitidine). For reference, the D50 for EPZ-5676 was 0.20 and 0.0097 μM in the MOLM-13 and MV4-11 cell lines, respectively. The D50az for azacitidine was 0.58 and 1.50 μM in the MOLM-13 and MV4-11 cell lines, respectively.

evidence for a role other chromatin regulators, such as HDACs, DNMTs, bromodomains, and LSD1, in the pathogenesis of MLL-r leukemia (Deshpande et al., 2012; Neff and Armstrong, 2013). We therefore studied the impact on MLL-r leukemia cell proliferation of combinations of EPZ-5676 with DNMT inhibitors, HDAC inhibitors, histone demethylase inhibitors, and bromodomain inhibitors. As summarized in Table 1, we observed a range of combination effects among these various inhibitors. With bromodomain and LSD1 inhibitors, we saw synergy or additivity, depending on the specific inhibitor used. In the case of HDAC inhibitors, we saw antagonism with EPZ-5676 in some cases and mere additivity in other cases (Table 1). These data suggest that combinations of EPZ-5676 and HDAC inhibitors are unlikely to provide greater benefit to MLL-r patients in a clinical setting. On the other hand, we saw consistent synergy between EPZ-5676 and DNMT inhibitors for selective inhibition of MLL-r leukemia cell proliferation. Further studies will be required to elucidate the detailed molecular mechanisms underlying these observations. In summary, the data presented here provide a rational foundation for combining EPZ-5676 with acute leukemia SOC chemotherapeutics for the treatment of MLL-r leukemia.


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