PLK1 Inhibitors Synergistically Potentiate HDAC Inhibitor Lethality in Imatinib Mesylate–Sensitive or –Resistant BCR/ABL+ Leukemia Cells In Vitro and In Vivo

Girija Dasmahapatra1, Hiral Patel1, Tri Nguyen1, Elisa Attkisson1, and Steven Grant1,2,3

Abstract

Purpose: To determine whether Polo-like kinase 1 (PLK1) inhibitors (e.g., BI2536) and histone deacetylase (HDAC) inhibitors (e.g., vorinostat) interact synergistically in the BCR/ABL+ leukemia cells sensitive or resistant to imatinib mesylate (IM) in vitro and in vivo.

Experimental Design: K562 and LAMA84 cells sensitive or resistant to imatinib mesylate and primary CML cells were exposed to BI2536 and vorinostat. Effects on cell viability and signaling pathways were determined using flow cytometry, Western blotting, and gene transfection. K562 and BV173/E255K animal models were used to test in vivo efficacy.

Results: Cotreatment with BI2536 and vorinostat synergistically induced cell death in parental or imatinib mesylate–resistant BCR/ABL+ cells and primary CD34+ bone marrow cells but was minimally toxic to normal cells. BI2536/vorinostat cotreatment triggered pronounced mitochondrial dysfunction, inhibition of p-BCR/ABL, caspase activation, PARP cleavage, reactive oxygen species (ROS) generation, and DNA damage (manifest by increased expression of γH2A.X, p-ATM, p-ATR), events attenuated by the antioxidant TBAP. PLK1 short hairpin RNA (shRNA) knockdown significantly increased HDACI lethality, whereas HDAC1–3 shRNA knockdown reciprocally increased BI2536-induced apoptosis. Genetic interruption of the DNA damage linker H1.2 partially but significantly reduced PLK1/HDAC inhibitor–mediated cell death, suggesting a functional role for DNA damage in lethality. Finally, BI2536/vorinostat cotreatment dramatically reduced tumor growth in both subcutaneous and systemic BCR/ABL+ leukemia xenograft models and significantly enhanced animal survival.

Conclusions: These findings suggest that concomitant PLK1 and HDAC inhibition is active against imatinib mesylate–sensitive or refractory CML and ALL cells both in vitro and in vivo and that this strategy warrants further evaluation in the setting of BCR/ABL+ leukemias.
Translational Relevance
The introduction of BCR/ABL kinase inhibitors, such as imatinib or dasatinib, resulted in improved treatment for patients with chronic myelogenous leukemia (CML) but many become refractory through various mechanisms, including mutations in kinase domain. Consequently, novel strategies are urgently needed to this disease. Recent evidence that Polo-like kinase 1 (PLK1) acts downstream of BCR/ABL makes it an attractive target for CML therapy, as it may be capable of circumventing resistance to BCR/ABL kinase inhibitors. The present findings indicate that histone deacetylase (HDAC) inhibitors, such as vorinostat, interact synergistically with a clinically relevant PLK1 inhibitor (BI2536) in imatinib mesylate–resistant cells both in vitro and in vivo through multiple mechanisms, raising the possibility that a combined HDAC/PLK1 inhibitor strategy may be of value in patients with CMLs resistant to standard approaches.

Materials and Methods

Cells
LAMA 84 cells were purchased from the German Collection of Microorganisms and Cell Cultures. K562 and Ba/F/3 cells were obtained as before (22). Cells were cultured in RPMI media as described previously (22). CD34+ cells were obtained with informed consent from patient bone marrows and processed as before (22). CML adult T315I and BV173/E255K ALL cells were generated as described (23). K562 cells expressing ectopically PLK1-CA or shRNA/scrambled sequence were generated by electroporation (Amaza) as described (24). K562 and LAMA84 cell lines were authenticated by STR DNA fingerprinting using the AmpFlSTR Identifier Kit (Applied Biosystems). The short tandem repeat (STR) profiles were compared with known American Type Culture Collection database and to the German Collection of Microorganisms and Cell Cultures database (http://www.dsmz.de/).

Reagents
PLK1 inhibitors BI2536 and BI6277 were purchased from ChemieTek Inc. and Selleck BioChem. GW843682 and 7-aminooxotinomycin D (7-AADD) were from Sigma-Aldrich and vorinostat was from Merck. All drugs were formulated in sterile dimethyl sulfoxide (DMSO) before use. Annexin V/propidium iodide (PI) was from BD PharMingen and MnTBAP was from Calbiochem.

Assessment of cell viability and apoptosis
Cell viability was monitored by flow cytometry using 7-AADD as before (24). Apoptosis was evaluated by Annexin V/PI staining (24) and verified by Wright–Giemsa staining. Results of morphologic assessment, 7-AADD staining, and Annexin V/PI staining were highly concordant.

Separation of S-100 fractions and assessment of cytochrome c release
Cells were harvested and cytosolic S-100 fractions were prepared as before (22, 24). Western blot analysis assessing cytochrome c, SMAC, and AIF release was conducted as below.

Immunoblot analysis
Immunoblotting was conducted as described previously (22, 24). Primary antibodies were as follows: AIF, cytochrome c, p-stat5, stat5, p-ATM, and ATR (Santa Cruz Biotechnology); p-BCR/ABL, BCR/ABL, p-PLK1(Thr210), PLK1, cleaved caspase-3, and p-ATR (Cell Signaling Technology); PARP (C-2–10, BioMol Research Laboratories); SMAC and γH2AX (Upstate Biotechnology); tubulin (Oncogene); ATM and Histone1.2 (Abcam); and p-PLK1 (Ser137; Millipore).
Measurement of ROS production

Cells were treated with 20 μmol/L 2′,7′-dichlorodihydrofluorescein diacetate for 30 minutes at 37°C, and fluorescence was monitored by flow cytometry and analyzed with CellQuest software (25).

Cell-cycle analysis

Cell-cycle distribution was determined by flow cytometry using a commercial software program (ModFit, Becton Dickinson) as per standard protocol (25).

Plasmids and shRNA

Plasmids encoding Homo sapiens PLK1 in pCMV6-Entry vectors were obtained from Origene Technologies. Four separate sequences were used to knock down PLK1 (i.e., 1- GGCAAGATTGCTTAAAGTCTCTGCTGCT, 2-ACCAGCAGTTCGATGATTTCCACCAGGCTT, 3-TACAGTCCCTCAATAAAGGCTTGAAGAAC, 4- TGGACTGGCAACCAAAGTCGATTGACG) and one nonspecific control sequence (NC- GGAATCTCATTCGATGCTAC) as negative control. Similarly, the following sequences are used to knock down Histone 1.2 (AAGGTTGCGAAGCCCAAGAAA, NC- GGAATCTCATTCGATGCTAC from SA Biosciences). Details of the shRNA for knocking down HDAC1–3 are follows (sh-HDAC1: 5′-GCTCCATCCGTCCAGATAACA-3′; sh-HDAC2: 5′-GCTGGAGCTGTGAAGTTAAAC3′; sh-HDAC3: 5′-GCACCATGCACAGAATTTGACCA-3′; NC- GGAATCTCATTCGATGCTAC).

Figure 1. Cotreatment with BI2536 and vorinostat synergistically induces cell death and dramatically reduces colony formation in K562 cell (A). K562 cells were treated with either BI2536 (1.0–6.0 nmol/L) or in combination with fixed vorinostat (1.5 μmol/L) concentrations for 48 hours. B, K562 cells were treated with vorinostat (0.5–2.0 μmol/L) in the presence or absence of fixed concentrations of BI2536 (5.0 nmol/L) for 48 hours. C, fractional effect (FA) values were determined by comparing results obtained for untreated controls and treated cells following exposure to agents administered at a fixed ratio (BI: vor = 1:300), after which median dose–effect analysis was used to characterize the nature of the interaction. Combination index (CI) values less than 1.0 denote a synergistic interaction. D, K562 cells were treated with BI2536 (5 nmol/L) / vorinostat (1.5 μmol/L) for 48 hours, after which cells were washed and plated in soft agar as described (23). Colonies, consisting of groups ≥ 50 cells, were scored at day 10. Values for each condition were expressed as a percentage of control colony formation. In all cases, values correspond to the means for triplicate determinations ± SD. A and B, *, significantly more than values for cells exposed to either BI2536 or vorinostat alone; P < 0.02; D, **, significantly less than values for cells exposed to either BI2536 or vorinostat alone; P < 0.01. For all studies, values represent the means for 3 experiments carried out in triplicate ± SD.
Transient transfections

Transient transfections of K562 cells used an Amaxa Nucleofector. Protocols for each cell line used Transfection Kit V and a cell-specific optimized protocol (T-16) as before (22).

Animal studies

Animal studies used Beige-nude-XID mice (NIH-III; Charles River). A total of 10 × 10^6 K562 cells were pelleted, washed twice with 1/2 PBS, and injected subcutaneously into the right flank. Once tumors were visible, 5 to 6 mice were treated with BI2536 with or without vorinostat and tumor growth or regression monitored as before (24, 25).

To investigate effects of tumor size on regimen efficacy, experiments were carried out with different initial tumor sizes [e.g., (i) average, 150 mm^3 and (ii) average, 550 mm^3].

Systemic tumor models used BV173/E255K/Luc cl4 cells as described earlier (23). Briefly, 2 × 10^6 BV173/E255K/Luc cl4 cells in 100 μL PBS were tail vein injected and animals noninvasively imaged using an in vivo Imaging System (IVIS-200; Xenogen) following luciferase injection (D-luciferin; Research Products International). BI2536 was administered orally by gavage; vorinostat was given intraperitoneally. Both drugs were given simultaneously daily, 3 days a week (TIW). BI2536 was dissolved in 0.1 (N) HCl solution; 0.9% NaCl was added as diluent. The BI2536 volume was 100 μL. Vorinostat was administered as described (24).

Statistical analysis

The significance of differences between experimental conditions was determined with the 2-tailed Student t test. Synergistic and antagonistic interactions were characterized using median dose–effect analysis in conjunction with a commercially available software program (CalcuSyn; ref. 26).

Results

HDACIs dramatically increase PLK1 lethality toward IM-sensitive or -resistant BCR/ABL+ leukemia cells but not normal CD34+ cells

Concomitant exposure (48 hours) of K562 cells to vorinostat (1.5 μmol/L) and very low, minimally toxic (e.g., 3 nmol/L) concentrations of BI, significantly increased apoptosis, which exceeded 80% at 5 to 6 nmol/L BI2536 (Fig. 1A). Vorinostat concentrations as low as 0.5 μmol/L, potentiated BI lethality which became more pronounced at concentrations ≥1.0 μmol/L (Fig. 1B). Time course analysis revealed discernible increases in cell death after 24 hours of exposure, which increased at 48 hours (Supplementary Fig. S1A). Median dose–effect analysis yielded combination

Figure 2. Co-exposure of parental K562 or imatinib-resistant BV173/E255 cells to BI2536 and vorinostat leads to modulation of BCR/ABL and stress-related pathways. K562 cells were treated with BI2536 (5.0 nmol/L) and vorinostat (1.5 μmol/L; A–D) for 24 hours. E, BV173/E255 cells were treated (24 hours) with BI2536 (3.0 nmol/L) and vorinostat (0.75 μmol/L). F, K562 cells were treated with BI2536 (5.0 nmol/L) and vorinostat (1.5 μmol/L) for the designated intervals. Expression of the indicated proteins was determined by Western blotting using the indicated antibodies. Each lane was loaded with 20 μg of protein; blots were stripped and reprobed with antibodies to tubulin to ensure equivalent loading and transfer. Results are representative of 3 independent experiments.
index values considerably below 1.0, reflecting a highly synergistic interaction (Fig. 1C). Combined treatment also strikingly reduced colony formation of K562 cells (Fig. 1D). In contrast, individual or combined treatment with BI and vorinostat minimally affected normal CD34+ cells (Supplementary Fig. S1B).

Similar interactions were observed when BCR/ABL+ LAMA84 were exposed to BI (2.0 nmol/L) and 1.0 μmol/L vorinostat (Supplementary Fig. S2A) or in K562 cells exposed to other PLK1 inhibitors (e.g., BI6277 or GW843682) or HDACIs (e.g., vorinostat or SBHA; Supplementary Fig. S2B). Median dose–effect analysis revealed synergism between GW843682 and vorinostat in LAMA84 cells (Supplementary Fig. S2C). Notably, BI2536/vorinostat synergism occurred in highly imatinib mesylate–resistant human T315I BCR/ABL+ ALL cells or E255K BV173 ALL cells bearing the E255K mutation (ref. 23; Supplementary Fig. S2D and S2E). In contrast to normal CD34+ cells, BI2536/vorinostat induced marked apoptosis in primary BCR/ABL+ CD34+ cells (Supplementary Fig. S2F). Finally, the BI2536/vorinostat regimen exerted pronounced lethality toward Ba/F3 cells expressing wild-type (wt) or 3 BCR/ABL mutants (E255K, M351T, or T315I; Supplementary Fig. S2G). In separate studies, sequential administration of BI2536 and vorinostat was slightly less effective than simultaneous exposure in inducing cell death (data not shown). These findings indicate that HDACIs synergistically potentiate the lethality of extremely low concentrations of PLK1 inhibitors in imatinib mesylate–sensitive or –resistant BCR/ABL+ leukemia cells but are relatively sparing toward their normal counterparts.

**HDACI/BI2536 co-administration enhances BCR/ABL inhibition and DNA damage**

Effects of vorinostat/BI2536 on various signaling and survival events were examined. These studies were conducted at relatively early exposure intervals and before the onset of extensive apoptosis (e.g., 24 hours). Combined treatment of K562 cells with BI2536 (5 nmol/L) and vorinostat (1.5 μmol/L) resulted in a marked increase in cytochrome c, AIF, and SMAC release into the cytosolic fraction, accompanied by enhanced PARP and caspase-3 cleavage (Fig. 2A and B). While the agents administered alone had little effect on BCR/ABL phosphorylation, combined treatment induced modest reductions in phospho-BCR/ABL expression but not total expression (Fig. 2B). Combined treatment had little effect on T210 phosphorylation of PLK1 but clearly reduced S137 phospho-PLK1. No changes in total PLK1 were noted. Finally, combined treatment reduced levels of phospho-STAT5, a downstream BCR/ABL target (27).

While vorinostat alone modestly upregulated histone 1.2, a marker of DNA damage (28), co-administration of BI2536, which had no effect by itself, dramatically increased histone 1.2 levels, accompanied by pronounced induction...
of γH2AX, an indicator of DNA double-strand breaks (ref. 29; Fig. 2C). Combined treatment also resulted in clear increases in phosphorylation of the checkpoint proteins ATR and ATM (Fig. 2C), accompanied by downregulation of DNA repair proteins [e.g., MLH1, MSH3, MSH6, MRE11, and RAD51; Fig. 2D]. Increased DNA damage occurred in highly imatinib mesylate–resistant BV173/E255 ALL cells (Fig. 2E). Finally, time course studies of K562 cells exposed to BI2536 and vorinostat resulted in clear increases in γH2A.X formation and ATM phosphorylation at 12 hours and ATR phosphorylation at 18 hours (Fig. 2F). In separate studies, co-administration of the broad caspase inhibitor BOC-FMK did not prevent DNA damage or ATM/ATR phosphorylation by the BI2536/vorinostat regimen (data not shown). Finally, histone 1.2 knockdown significantly reduced BI2536/vorinostat lethality (P < 0.05; Supplementary Fig. S3A) and diminished PARP cleavage (Supplementary Fig. S3B). Collectively, these findings indicate that the BI2536/vorinostat regimen downregulates various survival proteins and promotes DNA damage in BCR/ABL+ leukemia cells and argue for a functional role for DNA damage in lethality.

**PLK1 depletion increases HDACI lethality in BCR/ABL+ cells**

To evaluate the functional significance of PLK1 in these interactions, K562 cells ectopically expressing PLK1 were generated. Two clones (CA6 and CA9) expressing significantly greater levels of PLK1 than scrambled controls (Fig. 3A, inset) were used. The lethality of the BI2536 regimen in PLK1-expressing clones was significantly attenuated (P < 0.05) compared with controls (Fig. 3A). Conversely, shRNA knockdown of PLK1 (Fig. 3B, inset) significantly increased vorinostat lethality (Fig. 3B). Finally, PLK1-knockdown cells exposed to vorinostat exhibited significant increases in caspase-3 cleavage, γH2A.X formation, and ATM phosphorylation compared with controls (Fig. 3C).

**ROS generation by the BI2536/vorinostat regimen plays a significant functional role in lethality**

Previous evidence implicated oxidative injury in HDACI lethality toward myeloid leukemia cells (18, 30). While BI2536 and vorinostat alone modestly induced ROS, manifested as early as 30 minutes after administration and increasing over the ensuing 24 hours, combined treatment significantly increased ROS levels (Fig. 4A). Notably, the ROS scavenger TBAP blocked ROS generation (Fig. 4B) as well as lethality (Fig. 4C) in BI2536/vorinostat-treated cells. TBAP also blocked BI2536/vorinostat-mediated caspase-3 and PARP cleavage, γH2A.X formation, and ATM/ATR phosphorylation (Fig. 4D), suggesting a functional role for oxidative injury in DNA damage and lethality.
and accumulation of cells in G2–M may be due to inostat lethality.

Enhanced DNA damage may contribute to BI2536/vorinostat interactions. shRNA as described above in (A) and treated with BI2536 (5.0 nmol/L) for 24 hours, after which Western blot analysis was conducted to monitor expression of the indicated proteins. For A, *P < 0.05, significantly greater than values obtained for empty vector controls; P < 0.05.

Figure 5. Knocking down of HDAC1–3 by shRNA potentiates the lethality of BI2536 in K562 cells. A, K562 cells were transiently transfected with HDAC1–3 shRNA or scramble, respectively, and exposed (48 hour) to the indicated concentration of BI2536, after which cell death was monitored by 7-AAD staining and flow cytometry. Results represent the means ± SD for 3 separate experiments carried out in triplicate. Inset: Western blot analyses showing expression of HDACs expression in empty vector control and knockdown cells. B, cells were transfected with HDAC3 scrambled sequence or shRNA as described above in (A) and treated with BI2536 (5.0 nmol/L) for 24 hours, after which Western blot analysis was conducted to monitor expression of the indicated proteins. For A, *P < 0.05, significantly greater than values obtained for empty vector controls; P < 0.05.

HDAC knockdown increases BI2536 lethality in BCR/ABL leukemia cells

Previous studies have implicated HDACs (e.g., HDAC3) in the DNA damage response (31). Consequently, the effects of HDAC knockdown on responses to BI2536 were examined. Three transiently transfected lines exhibiting knockdown of HDAC1–3 respectively (Fig. 5A, inset) were significantly more susceptible to BI2536 lethality (2.5–7.5 nmol/L) than controls (Fig. 5A). Moreover, HDAC3 knockdown cells exposed to BI2536 displayed increased caspase-3 and PARP cleavage, γH2A.X formation, and phospho-ATR compared with controls (Fig. 5B). Similar results were obtained in HDAC1 or 2 knock-down cells (data not shown), arguing that interference with HDAC function contributes to BI2536/vorinostat anti-leukemic interactions.

Co-administration of BI2536 prevents vorinostat-induced G1 arrest and promotes G2–M accumulation

Exposure to vorinostat alone (24 hours) induced a marked increase in the G1 fraction, consistent with previous reports (refs. 32, 33; Supplementary Fig. S4A). BI2536 by itself modestly increased G2–M accumulation and slightly reduced the G1 fraction. However, vorinostat/BI2536 co-administration induced pronounced G2–M accumulation, accompanied by an increase in the subdiploid (apoptotic) population (Supplementary Fig. S4B). Virtually identical changes were observed in PK1 shRNA knockdown cells exposed to vorinostat but not in controls (Supplementary Fig. S4B and S4C), raising the possibility that abrogation of HDAC1-mediated G1 arrest and accumulation of cells in G2–M may be due to enhanced DNA damage may contribute to BI2536/vorinostat lethality.

The marked reduction in size of the flank tumors in BI2536/vorinostat-treated animals is shown in at the end

The BI2536/vorinostat regimen displays striking in vivo activity in early- and late-stage BCR/ABL xenograft models

To assess the in vivo activity of the BI2536 regimen, 2 xenograft models were used. In the first, Beige mice were injected in the flank with $10^6$ K562 cells; when tumors were first visible (volumes < $150 \text{ mm}^3$), animals were treated with BI2536 (30 mg/kg) ± vorinostat (70 mg/kg) for 2 weeks. Alternatively, identical treatment regimens were initiated when tumor volumes exceeded $550 \text{ mm}^3$. In the first model, vorinostat alone had little effect whereas BI2536 delayed but did not prevent tumor growth (Fig. 6A). However, combined treatment essentially abrogated tumors, which did not recur during the entire 38-day observation period. In the late-stage model, vorinostat alone had no effect, whereas BI2536 along marginally delayed tumor growth (Fig. 6B). However, combined treatment substantially reduced tumor size over the entire observation period. Western blot analysis of tumor sections obtained after 6 days of treatment (early-stage model) revealed that combined but not single-agent treatment downregulated phospho-BCR/ABL and downstream targets (phospho-STAT5 and -CRKL) while increasing PARP/caspase-3 cleavage and γH2A.X formation (Fig. 6C). Similar results were obtained in day-21 specimens (data not shown). Finally, BI2536 (30 mg/kg) and vorinostat (70 mg/kg) cotreatment significantly increased animal survival compared with no or single-agent treatment in a systemic imatinib-resistant luciferase-labeled BV173/E255K model (P < 0.05; Fig. 6D). Tumor weights for all treatment groups obtained after 6 days of treatment (early-stage model) revealed that combined but not single-agent treatment downregulated phospho-BCR/ABL and downstream targets (phospho-STAT5 and -CRKL) while increasing PARP/caspase-3 cleavage and γH2A.X formation (Fig. 6C). Similar results were obtained in day-21 specimens (data not shown).
of drug treatment (Supplementary Fig. S5A) and 14 days after terminating the treatment (Supplementary Fig. S5B). Finally, combined treatment substantially reduced tumor growth compared with single-agent treatment in a systemic, luciferase-labeled BV173/E255K model system (Supplementary Fig. S5C). Collectively, these findings indicate that combined in vivo treatment with BI2536 and vorinostat induces many of events observed in in vitro studies and that this regimen exhibits pronounced in vivo activity against early- and late-stage leukemia xenograft models.

Discussion

The present results indicate that HDACIs strikingly increase PLK1 inhibitor (e.g., BI2536) lethality in BCR/ABL+ leukemia cells, including those exhibiting marked resistance to imatinib mesylate through gatekeeper or other mutations. Previously, BI2536 showed single-agent activity against such cells and suggested that PLK1 represents an important BCR/ABL downstream target responsible for survival functions (13). Notably, HDACIs have been shown to enhance BCR/ABL kinase inhibitor activity through various mechanisms, including BCR/ABL down-regulation due to chaperone function disruption (20, 34) or enhanced BCR/ABL inhibition (35). Here, vorinostat/BI2536 promoted modest inactivation/dephosphorylation of BCR/ABL and its downstream target STAT5 (27). The mechanism underlying HDACI/BI2536-mediated disruption of the BCR/ABL pathway remains to be determined, as does the contribution of BCR/ABL inhibition to lethality. Indeed, preliminary studies in BCR/ABL-negative acute myelogenous leukemia (AML) cells suggest similar PLK1/HDAC inhibitor interactions.
(Dasmahapatra and Grant, unpublished observations). Such findings argue that BCR/ABL pathway inhibition is unlikely to be the sole mechanism of lethality for this regimen. Finally, vorinostat potentiated BI2536-mediated inhibition of PLK1 phosphorylation at Ser137, which has been implicated in late mitotic progression and the mitotic spindle checkpoint (36), potentially contributing to lethality.

The present results suggest that enhanced HDAC/PLK1 inhibitor lethality involves potentiation of DNA damage. HDACIs induce DNA damage through diverse mechanisms, including downregulation of DNA repair proteins (e.g., RAD51 and MRE11; ref. 17) or acetylation (e.g., Ku70; ref. 37). PLK1 also plays an important role in the mitotic spindle apparatus and DNA damage responses (38), suggesting that enhanced lethality stems from the disabling of the DNA damage and repair response. In support of this concept, combining BI2536 with vorinostat sharply increased expression of the atypical histone γH2A.X, reflecting double-strand DNA breaks (29) and DNA damage checkpoint kinase activation (ATM and ATR). Moreover, BI2536/vorinostat co-exposure produced reductions in the expression of multiple DNA repair proteins, including RAD51, MRE11, ERCC1, MSH2/6, and MLH1, compared with vorinostat alone. The mechanism underlying this phenomenon remains to be determined, but as in the case of BCR/ABL inactivation, may represent secondary, apoptosis-related events. In this context, knockdown of HDACIs (e.g., HDAC3) promotes DNA damage (31), possibly reflecting the contribution of HDACs to DNA repair complexes (39). Consistent with these findings, HDAC1–3 knockdown significantly increased BI2536-mediated DNA damage and lethality. Conversely, ectopic PLK1 expression significantly reduced BI2536/vorinostat-mediated apoptosis, whereas shRNA PLK1 knockdown significantly increased vorinostat-induced cell death, implicating on-target actions in synergistic interactions. Finally, the observation that knockdown of histone 1.2, which links DNA damage to the apoptotic apparatus (28) significantly protected cells, argues for a functional role for DNA damage in lethality.

HDACIs have been shown to kill transformed cells, including leukemia cells, through the induction of ROS (18), and selective induction of oxidative injury in transformed cells may reflect impairment in upregulation of antioxidant proteins (e.g., thioredoxin reductase; ref. 40). On the other hand, a link between PLK1 and oxidative injury has not previously been described. Interestingly, while minimally toxic vorinostat or BI2536 concentrations modestly induced ROS, combined treatment resulted in significant increases as early as 30 minutes after drug exposure which persisted over the ensuing 24 hours. The ability of the antioxidant TBAP to block BI2536/vorinostat-mediated ROS generation and lethality indicates an important functional role for oxidative injury in synergistic interactions. While ROS generation could stem from DNA damage (41), the observation that TBAP blocked γH2A.X formation and ATM/ATR activation suggests that ROS generation operates upstream of DNA breaks.

In cells sustaining DNA damage, HDACIs perturb the G1 checkpoint (42, 43), although interference with the intra-S (43) and G2–M checkpoints (43) has also been described. Here, minimally toxic concentrations of vorinostat induced marked G0–G1 arrest, consistent with the ability of this agent to upregulate p21cip1 (32). Exposure to BI2536 or PLK1 knockdown produced modest but discernible G2–M accumulation, reflecting the role of PLK1 in mitotic progression (44). Notably, vorinostat combined with BI2536 or PLK1 knockdown essentially abrogated G0–G1 arrest and resulted in a striking accumulation of cells in G2–M phase. Thus, combining HDAC with PLK1 inhibition may disable the G1 checkpoint while simultaneously increasing DNA damage, promoting accumulation of cells in G2–M and subsequently apoptosis.

The combination of BI2536 and vorinostat showed marked inhibition of tumor growth of imatinib mesylate–sensitive or -resistant BCR/ABL+ leukemias in both flank and systemic in vivo models and significantly increased survival compared with single agents. Notably, multiple events observed in cells exposed to these agents in vitro (e.g., BCR/ABL and STAT3 dephosphorylation, γH2A.X formation, and PARP cleavage occurred in vivo, suggesting that similar mechanisms may be operative in the latter setting. The relative lack of toxicity of this regimen was consistent with the minimal in vitro effects observed in normal hematopoietic cells (CD34+). Transformed cells exhibit impaired DNA damage checkpoints (45) and increased PLK1 expression in leukemia (10) may reflect a compensatory mechanism designed to circumvent otherwise lethal effects of DNA damage. Thus, leukemia cells may be particularly vulnerable to a strategy combining PLK1 inhibition with HDACIs, which promote DNA injury while disrupting checkpoint mechanisms (46). Of note, the BI2536/vorinostat regimen displayed significant activity against large tumors in immunocompromised mice. Such tumors tend to be resistant to both conventional and targeted therapy as a consequence of impaired vasculature, a hypoxic environment, and a low growth fraction (47). The ability of the BI 2536/vorinostat regimen to induce substantial tumor regression in advanced disease argues that resistance resulting from these mechanisms may be circumvented, at least in part, by the current strategy.

HDACIs such as vorinostat are approved for the treatment of cutaneous T-cell lymphoma (CTCL; ref. 48). However, while a role for these agents in CML has not yet been established, recent studies suggest that HDACIs may interact with tyrosine kinase inhibitors to target CML stem cells (49). In light of preclinical evidence of activity of PLK1 inhibitors in CML (13) and early preclinical indications of in vivo activity of such agents in other diseases (e.g., non-Hodgkin lymphoma; ref. 50), the concept of enhancing PLK1 inhibitor activity with HDACIs warrants further attention. Accordingly, studies investigating PLK1/HDAC inhibitor interactions in...
PLK1 Inhibitor and HDACi in CML Cells

BCR/ABL-negative hematopoietic malignancies are currently underway.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: G. Dasmahapatra, S. Grant Development of methodology: G. Dasmahapatra, S. Grant Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Dasmahapatra, H. Patel, T.K. Nguyen, E. Attkisson, S. Grant Analysis and interpretation of data (e.g., statistical analysis, bioinformatics; computational analysis): G. Dasmahapatra, H. Patel, T.K. Nguyen, S. Grant Writing, review, and/or revision of the manuscript: G. Dasmahapatra, S. Grant

References

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Dasmahapatra, T.K. Nguyen, E. Attkisson, S. Grant
Study supervision: G. Dasmahapatra, S. Grant

Grant Support
G. Dasmahapatra, H. Patel, and S. Grant were supported by awards CA63753, CA93738, and CA100866 from the NIH; award R0559-06 from the Leukemia and Lymphoma Society of America; the Multiple Myeloma Research Foundation, Myeloma Spore (P50CA142509), the V Foundation, and Lymphoma SPORE award 1P50 CA130805.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 28, 2012; revised October 26, 2012; accepted November 18, 2012; published OnlineFirst November 30, 2012.


PLK1 Inhibitors Synergistically Potentiate HDAC Inhibitor Lethality in Imatinib Mesylate–Sensitive or –Resistant BCR/ABL+ Leukemia Cells  In Vitro and In Vivo

Girija Dasmahapatra, Hiral Patel, Tri Nguyen, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-12-2799

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2012/11/30/1078-0432.CCR-12-2799.DC1

Cited articles
This article cites 50 articles, 26 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/19/2/404.full.html#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
/content/19/2/404.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.