PLK1 inhibitors synergistically potentiate HDAC inhibitor lethality in IM-sensitive or – resistant BCR/ABL⁺ leukemia cells in vitro and in vivo.

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ABSTRACT

**Purpose:** To determine whether PLK1 inhibitors (e.g. BI2536) and HDAC inhibitor (e.g. vorinostat) interact synergistically in CML cells sensitive or resistant to imatinib mesylate (IM) *in vitro* and *in vivo*.

**Experimental Design:** K562 and LAMA84 cells sensitive or resistant to IM 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:** Co-treatment with BI2536 and vorinostat synergistically induced cell death in parental or IM-resistant BCR/ABL*<sup>+</sup>* cells and primary CD34*<sup>+</sup>* bone marrow cells but was minimally toxic to normal cells. BI2536/vorinostat co-treatment triggered pronounced mitochondrial dysfunction, inhibition of p-BCR/ABL, caspase activation, PARP cleavage, ROS generation, and DNA damage (manifest by increased expression of γH2A.X, p-ATM, p-ATR), events attenuated by the anti-oxidant TBAP. PLK1 shRNA knockdown significantly increased HDACI lethality, whereas or HDAC 1-3 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 co-treatment dramatically reduced tumor growth in both subcutaneous and systemic BCR/ABL*<sup>+</sup>* leukemia xenograft models and significantly enhanced animal survival.

**Conclusions:** These findings suggest that concomitant PLK1 and HDAC inhibition is active against IM-sensitive or refractory CML cells both *in vitro* and *in vivo*, and that this strategy warrants further evaluation in the setting of BCR/ABL*<sup>+</sup>* leukemias.
Statement of translational relevance:

The introduction of BCR/ABL kinase inhibitors like imatinib or dasatinib resulted in improved treatment for CML patients, 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 HDAC inhibitors such as vorinostat interact synergistically with a clinically relevant PLK1 inhibitor (BI2536) in IM-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 CML patients resistant to standard approaches.
INTRODUCTION

Chronic myelogenous leukemia (CML) is a neoplastic stem cell disorder characterized by the 9;22 translocation, producing the BCR/ABL fusion protein, a constitutively active kinase which signals downstream to multiple anti-apoptotic proteins e.g., CRKL, STAT5, and Bcl-xL (1). CML treatment has been revolutionized by the BCR/ABL kinase inhibitor imatinib mesylate (IM) and second-generation agents e.g., nilotinib, dasatinib, and bosutinib (2, 3). Despite long-term responses to these agents, drug intolerance, the development of resistance-conferring mutations (e.g., T315I), and the failure to eradicate primitive leukemia-initiating cells represent therapeutic challenges (4). In addition, patients in blast phase of CML or with Ph1+ ALL generally respond poorly to therapy (5). Consequently, the need for new and more effective treatment options remains.

Polo-like kinase 1 (PLK1) is a conserved serine-threonine kinase involved in mitotic progression through interactions with cyclin B, the CDC25C phosphatase, and Wee1 (6). It has been implicated in progression into M-phase, mitotic spindle formation, cytokinesis, and chromosome segregation (7). PLK1 modulates DNA damage responses, including recovery from the G2 DNA damage checkpoint (8). Moreover, interactions between PLK1 and multiple checkpoint proteins, including Chk1/2, p53, clasin, and FoxM1 have been described (9). PLK1 is highly expressed in multiple malignancies, including leukemia (10) and lymphoma (11), prompting the development of multiple PLK1 inhibitors, including the specific ATP-competitive inhibitor BI 2536, which displays a 10,000-fold increase (12) in specificity for PLK1 compared to other tyrosine and threonine kinases (12). It was recently reported that PLK1 represented a downstream target of BCR/ABL in CML cells, and that PLK1 interruption by inhibitors such as BI 2536 or shRNA knockdown promoted leukemia cell death in highly IM-resistant cells expressing BCR/ABL gatekeeper mutations e.g., T315I (13). BI6727 (volasertib) is a highly potent, clinically relevant PLK1 inhibitor which has superior pharmacokinetic properties compared to BI2536 (14).

Histone deacetylase inhibitors (HDACIs) act by modifying chromatin structure, and by extension, gene expression (15). These agents also trigger acetylation of various non-histone...
proteins, particularly those implicated in DNA damage responses, including DNA repair proteins (Ku70) and chaperone proteins (Hsp90) (16). In addition, HDACIs down-regulate DNA repair proteins e.g., Rad51 and MRE11 (17). Indeed, HDACI lethality has been attributed to oxidative injury e.g., reactive oxygen species; ROS) (18), due impaired induction of anti-oxidant proteins (19). HDACIs have been shown to enhance the activity of tyrosine kinase inhibitors in CML cells (20), including early progenitor cells (21). Currently, no information is exists concerning PLK1/HDAC inhibitor interactions in human CML cells. Consequently, interplay between BI2536 and the HDACI vorinostat have been examined in BCR/ABL⁺ leukemia cells, including highly IM-resistant cells expressing gatekeeper mutations. The present results demonstrate highly synergistic interactions both in vitro and in vivo in IM-sensitive and –resistant BCR/ABL⁺-leukemia cells, and suggest multiple mechanisms, including enhanced inhibition of BCR/ABL and downstream targets, as well as marked potentiation of oxidative injury and DNA damage. These findings provide a theoretical foundation for a strategy combining HDAC and PLK1 inhibitors to eradicate BCR/ABL⁺ leukemia cells.
MATERIALS AND METHODS

Cells
LAMA 84 cells were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). K562, BaF/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 cells were generated as described (23). K562 cells expressing ectopically PLK1-CA or shRNA/scrambled sequence were generated by electroporation (Amaxa, GmbH, Germany) as described (24). K562 and Lama84 Cell lines were authenticated by STR DNA fingerprinting using the AmpFlSTR Identifiler kit (Applied Biosystems). The STR profiles were compared with known American Type Culture Collection (ATCC) data base and to the German Collection of Microorganisms and Cell Cultures database (http://www.dsmz.de/).

Reagents
PLK-1 inhibitors BI-2536 and BI-6277 were purchased from ChemieTek Inc (Indianapolis, IN) and Selleck BioChem (Houston TX). GW843682 and 7-AminoactinomycinD (7-AAD) were from Sigma-Aldrich (St Louis, MO); vorinostat was from Merck (Whitehouse Station, N.J). All drugs were formulated in sterile DMSO before use. Annexin V/PI was from BD PharMingen (San Diego, CA). MnTBAP was from Calbiochem (San Diego, CA).

Assessment of cell viability and apoptosis
Cell viability was monitored by flow cytometry using 7AAD (7-aminoactinomycin D) as before (24). Apoptosis was evaluated by Annexin V/PI staining (24) and verified by Wright-Giemsa Staining. Results of morphologic assessment, 7AAD 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 performed as below.
**Immunoblot Analysis**

Immunoblotting was performed as described previously (22, 24). Primary antibodies were as follows: AIF, cytochrome c, p-stat5, stat5, p-ATM, ATR: Santa Cruz Biotechnology, Santa Cruz, CA.; p-BCR/ABL, BCR/ABL, p-PLK1(Thr210), PLK1, Cleaved caspase-3, p-ATR: Cell Signaling Technology, Beverly, MA; PARP (C-2–10): BioMol Research Laboratories, Plymouth, MA; SMAC and γH2A.X: Upstate Biotechnology, Lake Placid, NY; Tubulin: Oncogene, San Diego, CA. ATM and Histone1.2: Abcam, Cambridge, MA. p-PLK1 (Ser137): Millipore, Billerica, MA.

**Measurement of ROS Production**

Cells were treated with 20μM 2',7'-dichlorodihydrofluorescein diacetate for 30min. at 37°C and fluorescence was monitored by flow cytometry and analyzed with Cell Quest 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 PLK-1 in pCMV6Entry vectors were obtained from Origene Technologies, Rockville, MD. Four separate sequences were employed to knock down PLK1 (i.e., 1- GGCAAGATTGTGCCTAAGTCTCTGCTGCT, 2- ACCAGCAGTCGTAGGATTCCAC-GGCTT, 3- TCACAGTCTCTCAATAAGGGCTTGGAGAAC, 4- TGGACTGGCAACCAAAG T-GGAATCTCATTCGATGCATAC) and one non-specific control sequence (NC- GGAATCTCATTCGATGCATAC) as negative control. Similarly, the following sequences are used to known down Histone 1.2 (AAGGTTCGAAGCGCAAGAAA, NC-GGAATCTCATTCGATGCATAC- from SA Biosciences, Frederick, MD). Details of the shRNA for knocking down HDAC1, 2 &3 are follows (shHDAC1; 5' GCTCCATCCGTCCAGATAACA 3’ shHDAC2; 5' GCTGGAGCTGTGAAGTTAAAC 3’ shHDAC3; 5’GCACCATCGCAAGAAGTTTAAG3’ NC- GGAATCTCATTCGATGCATAC ).
Transient Transfections

Transient transfections of K562 cells employed an Amaza Nucleofector (Cologne, Germany). Protocols for each cell line used transfection kit V and a cell-specific optimized protocol (T-16) as before (22).

Animal Studies

Animal studies utilized Beige-nude-XID mice (NIH-III; Charles River, Wilmington, MA, USA). 10x10^6 K562 cells were pelleted, washed twice with 1X PBS, injected subcutaneously into the right flank. Once tumors were visible, 5 to 6 mice were treated with BI 2536 ± vorinostat and tumor growth or regression monitored as before (24, 25). To investigate effects of tumor size on regimen efficacy, experiments were performed with different initial tumor sizes e.g., 1) average 150mm^3; 2) average 550 mm^3; Systemic tumor models employed BV173/E255K/Luc cl4 cells as described earlier (23). Briefly, 2 x 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). BI 2536 was administered orally by gavage; vorinostat was given IP. Both drugs were given simultaneously daily, three days a week (TIW). BI2536 was dissolved in 0.1 (N) HCl solution; 0.9% NaCl was added as diluant. The BI2536 volume was 100 uL. Vorinostat was administered as described (24).

Statistical Analysis

The significance of differences between experimental conditions was determined with the two-tailed Student t test. Synergistic and antagonistic interactions were characterized using Median Dose Effect analysis in conjunction with a commercially available software program (CalcuSyn, Biosoft, Ferguson, MO)(26) .
RESULTS

*HDACIs dramatically increase PLK-1 lethality toward IM-sensitive or –resistant BCR/ABL*+ leukemic cells but not normal CD34+ cells

Concomitant exposure (48 hr) of K562 cells to vorinostat (1.5 μM) and very low, minimally toxic (e.g., 3 nM) concentrations of BI, significantly increased apoptosis, which exceeded 80% at 5-6 nM BI2536 (Fig 1A). Vorinostat concentrations as low as 0.5 μM, potentiated BI lethality which became more pronounced at concentrations ≥ 1.0 μM (Fig 1B). Time course analysis revealed discernible increases in cell death after 24 hr of exposure, which increased at 48 hr (Supplemental Fig 1A). Median Dose Effect analysis yielded Combination 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 (Supplemental Fig 1B).

Similar interactions were observed when BCR/ABL+ LAMA84 were exposed to BI (2.0 nM) and 1.0 μM vorinostat (Supplemental Fig 2A), or in K562 cells exposed to other PLK1 inhibitors (e.g., BI 6277 or GW843682) or HDACIs (e.g. vorinostat or SBHA) (Supplemental Fig 2B). Median Dose Effect analysis revealed synergism between GW 843682 and vorinostat in LAMA84 cells (Supplemental Fig 2C). Notably, BI2536/vorinostat synergism occurred in highly IM-resistant human T315I BCR/ABL+ ALL cells or E255K BV173 cells bearing the E255K mutation (23) (Supplemental Fig 2D and 2E). In contrast to normal CD34+ cells, BI2536/vorinosatat induced marked apoptosis in primary BCR/ABL+CD34+ cells (Supplemental Fig 2F). Finally, the BI2536/vorinostat regimen exerted pronounced lethality toward BaF/3 cells expressing wt or three BCR/ABL mutants (E255K, M351T, or T315I; Supplemental Fig 2G). In separate studies, sequential administration of BI2536 and vorinostat were 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 IM-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 performed at relatively early exposure intervals and before the onset of extensive apoptosis (e.g., 24 hrs). Combined treatment of K562 cells with BI2536 (5 nM) and vorinostat (1.5 μM) 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-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 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 up-regulated histone 1.2, a marker of DNA damage (28), co-administration of BI-2536, which had no effect by itself, dramatically increased histone 1.2 levels, accompanied by pronounced induction of γH2A.X, an indicator of double-strand DNA breaks (29) (Fig 2C). Combined treatment also resulted in clear increases in phosphorylation of the checkpoint proteins ATR and ATM (Fig 2C), accompanied by down-regulation of DNA repair proteins (e.g., MLH1, MSH3, MSH6, MRE11, and RAD51; Fig 2D). Increased DNA damage occurred in highly IM-resistant BV173/E255 ALL cells (Fig 2E). Finally, time course studies of K562 cells exposed to BI-2536 and vorinostat resulted in clear increases in γH2A.X formation and ATM phosphorylation at 12 hr, and ATR phosphorylation at 18 hr (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 BI-2536/vorinostat regimen (data not shown). Finally, histone 1.2 knock-down significantly reduced BI-2536/vorinostat lethality (P <0.05; Supplemental Fig 3A), and diminished PARP cleavage (Supplemental Fig 3B). Collectively, these findings indicate that the BI-2536/vorinostat regimen down-regulates 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 employed. The lethality of the BI-2536 regimen in PLK1-expressing clones was significantly attenuated (P < 0.05) compared to controls (Fig 3A). Conversely, shRNA knock-down of PLK1 (Fig 3B, inset) significantly increased vorinostat lethality (Fig 3B). Finally, PLK1 knock-down cells exposed to vorinostat exhibited significant increases in caspase-3 cleavage, γH2A.X formation, and ATM phosphorylation compared to controls (Fig 3C).

**ROS generation by the BI-2536/vorinostat regimen plays a significant functional role in lethality**

Previous evidence implicated oxidative injury in HDACI lethality toward myeloid leukemia cells (18, 30). While BI-2536 and vorinostat alone modestly induced ROS, manifested as early as 30 min after administration and increasing over the ensuing 24 hr, 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 BI-2536/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.

**HDAC knock-down increases BI-2536 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 knock-down on responses to BI-2536 were examined. Three transiently transfected lines exhibiting knock-down of HDACs 1, 2, or 3 respectively (Fig 5A, inset) were significantly more susceptible to BI-2536 lethality (2.5-7.5 nM) than controls (Fig 5A). Moreover, HDAC3 knock-down cells exposed to BI-2536 displayed increased caspase-3 and PARP cleavage, λH2A.X formation, a phospho-ATR than controls (Fig 5B). Similar results were obtained in HDAC1 or HDAC2 knock-down cells (data not shown), arguing that interference with HDAC function contributes to BI-2536/vorinostat anti-leukemic interactions.
**Co-administration of BI-2536 prevents vorinostat-induced G₁ arrest and promotes G₂M accumulation**

Exposure to vorinostat alone (24 hr) induced a marked increase in the G₁ fraction, consistent with previous reports (32, 33) (Supplemental Fig 4A). BI-2536 by itself modestly increased G2M accumulation and slightly reduced the G₁ fraction. However, vorinostat/BI2536 co-administration induced pronounced G₂M accumulation, accompanied by an increase in the sub-diploid (apoptotic) population (Supplemental Fig 4A). Virtually identical changes were observed in PLK1 shRNA knock-down cells exposed to vorinostat, but not in controls (Supplemental Fig 4B-C), raising the possibility that abrogation of HDACI-mediated G₁ arrest and accumulation of cells in G₂M may be due to enhanced DNA damage contribution to BI-2536/vorinostat lethality.

**The BI-2536/vorinostat regimen displays striking in vivo activity in early- and late-stage BCR/ABL⁺ xenograft models.**

To assess the in vivo activity of the BI-2536 regimen, two xenograft models were employed. In the first, Beige mice were injected in the flank with 10 x 10⁶ K562 cells; when tumors were first visible (volumes less than 150 mm³), animals were treated with BI-2536 (30 mg/kg) ± vorinostat (70 mg/kg) for 2 weeks. Alternatively, identical treatment regimens were initiated when tumor volumes exceeded 550 mm³. In the first model, vorinostat alone had little effect, whereas BI-2536 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 BI-2536 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 down-regulated 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 (30mg/kg) and vorinostat (70mg/kg) co-treatment significantly increased animal survival compared to 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 did not decline by more than 10%, and no other signs of toxicity (torpor, weight loss etc.) were observed (data not shown).

The marked reduction in size of the flank tumors in BI-2536/vorinostat-treated animals is shown in at the end of drug treatment (Supplemental Fig 5A) and 14 days after terminating treatment (Supplemental Fig 5B). Finally, combined treatment substantially reduced tumor growth compared to single-agent treatment in a systemic, luciferase-labeled BV173/E255K model system (Supplemental Fig 5C). Collectively, these findings indicate that combined in vivo treatment with BI-2536 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 IM through gatekeeper or other mutations. Previously, BI2536 demonstrated 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 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 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 down-regulation of DNA repair proteins (e.g., RAD51, MRE11) (17) or acetylation (e.g., Ku70) (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 BI 2536 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, BI 2536/vorinostat co-exposure produced reductions in the expression of multiple DNA repair proteins, including RAD51, MRE11, ERCC1, MSH2/6, and MLH1, compared to 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, knock-down of HDACIs (e.g., HDAC3) promote DNA damage (31), possibly reflecting the
contribution of HDACs to DNA repair complexes (39). Consistent with these findings, HDACs 1-3 knock-down significantly increased BI 2536-mediated DNA damage and lethality. Conversely, ectopic PLK1 expression significantly reduced BI 2536/vorinostat-mediated apoptosis whereas shRNA PLK1 knock-down significantly increased vorinostat-induced cell death, implicating on-target actions in synergistic interactions. Finally, the observation that knock-down 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 up-regulation of antioxidant proteins (e.g., thioredoxin reductase) (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 induce ROS, combined treatment resulted in significant increases as early as 30 min after drug exposure which persisted over the ensuing 24 hr. The ability of the antioxidant TBAP to block BI 2536/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 G2M checkpoints (43) have also been described. Here, minimally toxic concentrations of vorinostat induced marked G0/G1 arrest, consistent with the ability of this agent to up-regulate p21CIP1 (32). Exposure to BI 2536 or PLK1 knockdown produced modest but discernible G2M accumulation, reflecting the role of PLK1 in mitotic progression (44). Notably, vorinostat combined with BI 2536 or PLK1 knockdown essentially G0/G1 arrest, and resulted in a striking accumulation of cells in G2M. Thus, combining HDAC with PLK1 inhibition may disable the G1 checkpoint while simultaneously increasing DNA damage, promoting accumulation of cells in G2M and subsequently apoptosis.

The combination of BI 2536 and vorinostat demonstrated marked inhibition of tumor growth of IM-sensitive or –resistant BCR/ABL+ leukemias in both flank and systemic in vivo models, and significantly increased survival compared to single agents. Notably, multiple events
observed in cells exposed to these agents in vitro (e.g., BCR/ABL and STAT5 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 BI 2536/vorinostat regimen displayed significant activity against large tumors in immune-compromised 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 CTCL (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 pre-clinical evidence of activity of PLK1 inhibitors in CML (13), and early pre-clinical indications of in vivo activity of such agents in other diseases (e.g. non-Hodgkin’s lymphoma) (50), the concept of enhancing PLK1 inhibitor activity with HDACIs warrants further attention. Accordingly, studies investigating PLK1/HDAC inhibitor interactions in BCR/ABL-negative hematopoietic malignancies are currently underway.
Legend

Figure 1: Co-treatment 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 nM) or in combination with fixed vorinostat (1.5µM) concentrations for 48 hr. (B) K562 cells were treated with vorinostat (0.5 -2.0 µM) in the presence or absence of fixed concentrations of BI2536 (5.0 nM) for 48 hr. (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 employed to characterize the nature of the interaction. Combination Index (C.I.) values less than 1.0 denote a synergistic interaction. (D) K562 cells were treated with BI2536 (5 nM) ± vorinostat (1.5µM) for 48 hrs, 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 ± S.D. A-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 performed in triplicate ± S.D.

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 nM) ± vorinostat (1.5µM) (A-D) for 24 hr. (E) BV173/E255 cells were treated (24 hr) with BI2536 (3.0 nM) ± vorinostat (0.75µM). (F) K562 cells were treated with BI2536 (5.0 nM) ± vorinostat (1.5µM) 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 re-probed with antibodies to tubulin to ensure equivalent loading and transfer. Results are representative of three independent experiments.
Figure 3: PLK-1 protein expression plays a significant functional role to potentiate the lethality of vorinostat in K562 cells.

K562 cells were stably transfected with (A) PLK-1-CA or empty vector (pCMV6 Entry) cDNA (*Inset*: western blots showing expression of PLK1 expression in empty vector control and overexpressing cells) and exposed (48 hr) to BI2536 (6.0 nM) ± vorinostat (1.25 µM), (B) shPLK1 or empty vector control pRFP-C-RS cDNA and exposed (48 hr) to indicated concentration of vorinostat, after which cell death was monitored by 7-AAD staining and flow cytometry (*Inset*: western blots showing expression of PLK1 expression in empty vector control and knockdown cells). Results represent the means ± S.D. for 3 separate experiments performed in triplicate. (C) K562 cells expressing either shPLK1 or empty vector control pRFP-C-RS cDNA as (B) above and treated with vorinostat (1.5µM) for 24 hrs, after which western blot analysis was performed to monitor expression of the indicated proteins. For A and B, * = significantly greater than values obtained for empty vector controls; P < 0.05.

Figure 4: Combined BI2536/vorinostat treatment robustly induces ROS generation which is circumvented by anti-oxidants.

(A) K562 cells were treated with BI2536 (5.0 nM) ± vorinostat (1.5 µM) after which ROS generation was monitored at the indicated intervals (B) K562 cells were treated with BI2536 (5.0 nM) ± vorinostat (1.5µM) ± pre-treatment (3 h) with 400 µM TBAP for 24 hrs, after which ROS generation was monitored as in Methods. (C) After treatment of K562 cells as in (B) for 48 hrs, cell death was monitored by 7AAD staining (D). Following 24 hr of drug exposure as in (C) above, expression of the indicated proteins was monitored by western blotting. Blots were stripped and re-probed with anti-tubulin antibodies to ensure equal loading and transfer of protein (20 µg each lane). For A* = significantly different from values for single drug treatment and C, * = significantly different from values for combination treatment without TBAP pretreatment controls; P < 0.05.
Figure 5: Knocking down of HDAC1, HDAC2 & HDAC3 by shRNA potentiates the lethality of BI2536 in K562 cell

(A) K562 cells were transiently transfected with HDAC1, HDAC2, HDAC3 shRNA or scramble respectively, and exposed (48 hr) to indicated concentration of BI2536, after which cell death was monitored by 7-AAD staining and flow cytometry. Results represent the means ± S.D. for 3 separate experiments performed in triplicate. Inset: Western blots 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 nM for 24 hrs, after which western blot analysis was performed to monitor expression of the indicated proteins. For A, * = significantly greater than values obtained for empty vector controls; P < 0.05

Figure 6: The BI-2536/vorinostat regimen displays striking in vivo activity in BCR/ABL+ xenograft models

Beige mice were injected in the flank with 10 x10^6 K562 cells. Once tumors formed, mice were divided into two groups with average tumor sizes of approximately (A) 150 mm^3 or (B) 550 mm^3 and treated with the designated doses of BI2536 ± vorinostat thrice weekly as in Methods or as indicated for 14 days or 24 days respectively. Tumor volumes were measured twice weekly and mean tumor volumes were plotted against days of treatment. Tumor samples were extracted from mice after (C) 6 days (early stage model) and of drug treatment, and lysed with lysis buffer followed by sonication. Western blotting was performed using the extracted proteins, which were probed with the indicated primary antibodies. Each lane was loaded with 20 μg of protein; blots were subsequently stripped and reprobed with antibodies to tubulin to ensure equivalent loading and transfer. (D) Survival curves of individual groups with systemically IM-resistant BV173/E255K tumors after 24 days following treatment with BI2536 (30mg/kg) ± vorinostat (70mg/kg) TIW. Control-blue, BI2536- green, vorinostat - yellow, BI2536 + vorinostat - purple) survival was evaluated from the first day of treatment until death using Kaplan–Meier analysis (* = P < 0.05).
Reference List

(1) Druker BJ. Translation of the Philadelphia chromosome into therapy for CML. Blood 2008;112:4808-17.


Figure 1
Figure 2

A

B

C

K562

Histone1.2 (s-100) fraction
γH2A.x
p-ATR
ATR
p-ATM
ATM
Tubulin

D

MLH1
MSH2
MSH6
ERCC1
MRE11
RAD51
Tubulin

E

BV173/E255K

F

K562

BI+vor

0 12 18 24 30

BI

0 12

vor

γH2A.x
Tubulin

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 3

% Cell Death

0 25 50 75 100

- K562-pCMV6 Entry
- K562-PLK1-CA-CL9
- K562-PLK1-CA-CL9

A

% Cell Death

0 25 50 75 100

- K562-pRFP-C-RS
- K562-shPLK1-CL9
- K562-sh PLK1

B

Figure 3

Vorinostat (µM)

0.00 1.25 1.50 2.00 3.00

- K562-pRFP-C-RS
- K562-shPLK1-CL9

C

CF caspase 3

P - ATM

p-tubulin

K562-pRFP-C-RS

K562-shPLK1-CL9

Cont

vor

Cont

vor
Figure 4
Figure 5

% Cell Death

A

Cont

BI-2.5nM

BI-5.0nM

BI-7.5nM

K562-scramble

K562-shHDAC1

K562-shHDAC2

K562-shHDAC3

HDAC1

HDAC2

HDAC3

B

Tubulin

pAkt

pH2AX

CF caspase 3

CF PARP

cont

scramble

BI

K562

shHDAC3

cont

BI

K562-shHDAC3
Figure 6

A. Tumor volume (mm$^3$) over days of treatment and no treatment for K562 cell lines treated with control, BI-30 mg/kg, vor-70 mg/kg, and BI+vor.

B. Similar data for K562 cell lines treated with control and BI+vor.

C. Western blot analysis of K562 tumor samples showing protein levels of p-Stat5, p-BCR/ABL, p-CrkL, γH2A.X, PARP, and CF Caspase 3.

D. Cumulative survival curves for BV173/E255K, showing a significant difference with P < 0.05.
Clinical Cancer Research

PLK1 inhibitors synergistically potentiate HDAC inhibitor lethality in IM-sensitive or -resistant BCR/ABL+ leukemia cells in vitro and in vivo.

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

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