The Novel Chk1 Inhibitor MK-8776 Sensitizes Human Leukemia Cells to HDAC Inhibitors by Targeting the Intra-S Checkpoint and DNA Replication and Repair

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Abstract

Interactions between the novel Chk1 inhibitor MK-8776 and the histone deacetylase (HDAC) inhibitor (HDACI) vorinostat were examined in human leukemia cells harboring wild-type (wt) or deficient p53. MK-8776 synergistically potentiated vorinostat-mediated apoptosis in various p53-wt or -deficient leukemia cell lines, whereas p53 knockdown by short hairpin RNA (shRNA) sensitized p53-wt cells to lethality of this regimen. Leukemia cell lines carrying FLT3-ITD were also sensitive to the MK-8776/vorinostat regimen. Synergistic interactions were associated with inhibition of Chk1 activity, interference with the intra-S-phase checkpoint, disruption of DNA replication, and downregulation of proteins involved in DNA replication (e.g., Cdt1) and repair (e.g., ChIP and BRCA1), resulting in sharp increases in DNA damage, reflected by enhanced γ-H2AX formation, and apoptosis. Moreover, leukemia cells expressing kinase-dead Chk1 (D130A) or Chk1 shRNA were significantly more sensitive to HDACIs compared with their wt counterparts and displayed downregulation of Chk2 phosphorylation following HDACI exposure. Finally, the MK-8776/vorinostat regimen was active in primary acute myelogenous leukemia (AML) blasts, particularly against the CD34+CD38−/CD0+/CD123+ population enriched for leukemia-initiating cells. In contrast, identical regimens were relatively sparing toward normal cord blood CD34+ cells. Together, these findings indicate that the novel Chk1 inhibitor MK-8776 markedly potentiates HDACI lethality in leukemia cells displaying various genetic backgrounds through mechanisms involving disruption of the intra-S checkpoint, DNA replication, and DNA repair. They also argue that leukemic cells, including those bearing oncogenic mutations associated with poor prognosis, for example, p53 deletion/mutation or FLT3-ITD, may also be susceptible to this strategy. Mol Cancer Ther; 12(6); 1–12. ©2013 AACR.

Introduction

Histone deacetylase inhibitors (HDACI) modify chromatin structure and by extension, gene expression, leading to cell death or differentiation (1). They also kill transformed cells through additional mechanisms (1), including reactive oxygen species (ROS)-related oxidative injury, chaperone (e.g., Hsp90) function disruption, and proapoptotic protein upregulation (e.g., Bim), among others. Recently, attention has focused on HDACI-mediated interference with the DNA damage checkpoint/repair machinery (1). Although HDACIs induce DNA damage (e.g., double-strand breaks; DSB; ref. 2), HDACI-mediated downregulation of checkpoint and/or repair proteins (3) may cooperate with direct actions (e.g., oxidative damage of DNA; ref. 4) to promote cell death.

The DNA damage response (DDR) is a versatile response to genomic DNA damage. It consists of cell-cycle checkpoints, DNA repair, a transcriptional program, and apoptosis (5). A critical function is to ensure cell-cycle arrest via checkpoint activation following DNA damage, permitting repair of lesions, or triggering apoptosis if they are irreparable, thereby preserving genomic integrity (5). DNA damage activates distinct, albeit overlapping and cooperating checkpoint pathways, which block S-phase entry (the G1–S or G1 checkpoint), delay S-phase progression (the S-phase checkpoint), or prevent mitotic entry (the G2–M–phase checkpoint; ref. 5). Checkpoints are activated via signaling networks consisting of sensors (multiprotein complexes, e.g., MRN and 9-1-1), proximal transducers [phosphoinositide 3-kinase (PI3K)-like kinases, e.g., ATM and ATR], signal amplifiers/mediators (e.g., BRCA1, claspin, FANCD2, etc.), distal transducers (i.e., Chk1 and Chk2), and effector proteins including Cdc25s, cdc2/Cdk1, Cdk2, p53, and p21CIP1 (5). In general, Chk1 is activated by ssDNA via ATR, whereas Chk2...
is activated by DSBs through ATM (5). Aside from checkpoint responses to exogenous genotoxic insults (e.g., chemotherapy or ionizing radiation), the ATR/Chk1-mediated intra-S-phase checkpoint pathway plays a central role in monitoring chromosome replication (designated the DNA replication checkpoint) and governing the S-phase DNA repair machinery (specifically homologous recombination; ref. 6) to ensure accurate genomic DNA duplication. These findings provide a theoretical basis for the notion that Chk1 inhibition [e.g., by Chk1 inhibitors or short hairpin RNA (shRNA)] itself induces DNA damage by disrupting DNA replication (7). Because transformed cells exhibit defective checkpoints (5), the DDR (and specifically Chk1) have become attractive targets for therapeutic intervention. Numerous Chk1 inhibitors have recently been developed to enhance the activity of DNA-damaging agents or radiation (5). MK-8776 (formerly SCH900776; ref. 8) is a novel specific Chk1 inhibitor currently under clinical development in combination with DNA-damaging agents (e.g., cytarabine) in acute myelogenous leukemia (AML; ref. 9).

Currently, little information exists about the impact of checkpoint disruption (particularly the intra-S-phase checkpoint) on HDACI responses of transformed cells. Recent evidence suggests that the HDACI vorinostat triggers marked alterations in DNA replication culminating in DNA damage in transformed cells (10). The ability of HDAC and Chk1 inhibitors to target DNA replication raises the possibility that Chk1 inhibitors might promote HDACI activity via disruption of the intra-S-phase checkpoint. To test this possibility, effects of MK-8776, a selective Chk1 inhibitor that targets the replication checkpoint (11), on vorinostat lethality have been examined in human leukemia cells, including those carrying wild-type (wt) or mutant p53, an important determinant of Chk1 inhibitor sensitivity (12). The present results indicate that MK-8776 synergistically increases vorinostat lethality in leukemia cells, including those bearing defective p53 or expressing FLT3-ITD, in association with disruption of the intra-S checkpoint and multiple proteins involved in DNA replication, DSB end-resection, and homologous recombination repair. They also suggest that this strategy may selectively target leukemia cells, including primitive progenitors enriched for leukemia-initiating cells.

Materials and Methods

Cells and cell culture

Human leukemia cell lines U937 [myelomonocytic M4-M5], histiocytic, p53-null (13), FLT3-wt (14), MV4-11 [myelomonocytic M5, biphenotypic, p53 mutated (15), FLT3-ITD; ref. 14], and MOLM-13 [monocytic M5a, p53-wt (16), FLT3-ITD (14) were purchased from American Type Culture Collection (ATCC) and maintained as previously described (17). OCI-AML-3 (myeloid M4, p53-wt; ref. 18) and FLT3-wt (14) cell lines were obtained from DSMZ and maintained in α-minimum essential medium containing 20% FBS. These cells were authenticated using basic short tandem repeat profiling within 6 months at the end of the studies. Experiments used logarithmically growing cells (3–6 × 10^5 cells/mL).

Bone marrow or peripheral blood samples were obtained with informed consent from patients with histologically documented AML undergoing routine diagnostic procedures with Virginia Commonwealth University Institutional Review Board approval (#HM 12517). Mononuclear cells were isolated and characterized (e.g., blasts >70% and viability > 95%) as described previously (19). All experiments were carried out at density of 1 × 10^6 cells/mL. Normal human cord blood CD34+ cells were isolated as previously described (19).

Drugs and chemicals

The selective Chk1 inhibitor MK-8776 and the panHDAC inhibitor vorinostat [formerly suberyanilide hydroxamic acid (SAHA)] were provided by Merck. The structures of MK-8776 and vorinostat are shown in Supplementary Fig. S1A. Suberohydroxamic acid (SBHA) was purchased from Calbiochem. Reagents were formulated in dimethyl sulfoxide (DMSO) and stored at −20°C. Stock solutions were diluted with serum-free RPMI medium to ensure final DMSO concentrations were less than 0.1%.

Flow cytometry

Apoptosis was evaluated by flow cytometry using Annexin V–FITC/PI staining as described previously (20). In some cases, loss of mitochondrial membrane potential and cell death were assessed by double staining with 3,3-dihexyloxacarbocyanine (DiOC6) and 7-AAD as before (20).

For analysis of the CD34+/CD38−/CD123+ population, mononuclear cells isolated from AML patient bone marrows were blocked by TruStain FcX on ice for 10 minutes, stained with C34-PE, CD38-PE/Cy7, and CD123-APC (Biolegend) on ice for 30 minutes followed by staining with Annexin V–FITC and 7-AAD at room temperature in the dark for 15 minutes. The percentage of apoptotic (Annexin V+) cells in the CD34+/CD38−/CD123+ population was determined using a FACSCanto flow cytometer (BD Biosciences).

Cell-cycle analysis of DNA content by propidium iodide (PI) staining was conducted by flow cytometry using Modfit LT2.0 software, as described previously (21). Click-iT Edu CellCycle 488-Red (7-AAD) Assay Kit (Invitrogen) was used to monitor DNA replication via incorporation of the thymidine analog 5-ethyl-2-deoxyuridine (EdU) into genomic DNA during DNA synthesis.

Plasmids, shRNA, and transfection

Wild-type (Chk1wt) and kinase-dead Chk1 (Chk1KD, D130A), cloned into the pEFYP-N1 vector, were provided by Dr. Yolanda Sanchez (Dartmouth College, Hanover, NH; ref. 22). SureSilencing shRNA plasmids (neomycin resistance) were purchased from SABioscience, including...
shChk1 (human CHEK1, GTGAAAGTTGGCTATCAAT), shTP53 (human TP53, GGAAGCTCATGGTAATCT), and negative control shRNA (shNC, GGAGTCTCATTGGATGATAC). U937 or OCI-AML-3 cells were stably transfected with these constructs using the Amaxa Nucleofector (Amaxa GmbH), and clones expressing GFP-Chk1 or downregulated Chk1 or TP53 were selected by G418 (400 μg/mL).

**Immunoblotting**

Samples from whole-cell lysates were prepared and 30 μg of protein per condition were subjected to Western blot analysis as previously described (17). Blots were reprobed with anti-β-actin (Sigma) or anti-α-tubulin (Oncogene Inc.) to ensure equal protein loading. Primary antibodies included: anti-p53, anti-phospho-cdc2 (Tyr15/Thr14), anti-phospho-Cdk2 (Tyr15/Thr14), anti-Cdt1, and anti-Chk1 (Santa Cruz Biotechnology); anti-p21/WAF1/CIP1 (Transduction Laboratories); anti-cleaved caspase-9 (Asp315), anti-cleaved caspase-3 (Asp175), anti-Chk1, anti-phospho-Chk1 (S296), anti-phospho-BRCA1 (Ser1524), anti-Chk2, anti-phospho-Chk2 (Thr68), anti-phospho-p53 (Ser15), and anti-phospho-p53 (Ser20; Cell Signaling); anti-PARP (Biomol); anti-phospho-p53 (Ser15), and anti-phospho-p53 (Ser20; Cell Signaling); and anti-BRCA1 (Ser1423), anti-acetylated α-tubulin (K40; Sigma); anti-acetylated histone H3, and anti-phospho-histone H2AX (Ser139; Millipore).

**Statistical analysis**

Values represent the means ± SD for at least 3 independent experiments carried out in triplicate. The significance of differences between experimental variables was determined using the Student t test or one-way ANOVA with Tukey–Kramer multiple comparisons test. The significance of P values was less than 0.05 (*), 0.01 (**), or 0.001 (***). Wherever indicated.

**Results**

**MK-8776 interacts synergistically with HDACIs in both p53 wild-type and deficient leukemia cells**

Responses to Chk1 inhibitors, including MK-8776, combined with DNA-damaging agents (12) or radiation (23) largely depend upon p53 status, with p53-deficient tumor cells more sensitive than p53-wt cells (8). Effects of MK-8776 on leukemia cells harboring either wt or deficient p53 were first examined. Leukemia cells carrying wt p53 [e.g., OCI-AML-3 (18) and MOLM-13 (16)] exhibited moderate p53 expression, whereas those bearing mutant p53 [e.g., MV-4-11 cells that carry point mutations at codon 344 (15)] had higher p53 expression (Fig. 1A, top). Expression of p53 was not detected in U937 cells, which is functionally p53 null due to a large deletion in the p53 gene (13). As shown in Fig. 1A, bottom, sensitivities to MK-8776 varied in different cell lines. MV-4-11 and MOLM-13 cell lines, both harboring the FLT3-ITD mutation, which is frequently observed in AML (14), were relatively more sensitive to MK-8776 than U937 and OCI-AML-3 cells, which do not carry FLT3-ITD (14).

Coadministration of minimally toxic concentrations of MK-8776 with vorinostat or SBHA significant increased lethality in all lines, although effects were less pronounced in OCI-AML-3 cells bearing wt-p53 but without FLT3-ITD (Fig. 1B). Median dose effect analysis yielded combination index (CI) values substantially less than 1.0, indicating synergism (Supplementary Table S1; CI value ≤0.40 in U937, ≤0.25 in MV-4-11, ≤0.75 in OCI-AML-3, and ≤0.70 in MOLM-13), including in MK-8776-resistant OCI-AML-3 cells (Fig. 1A and Supplementary Fig. S1B). Synergism between MK-8776 and vorinostat was also observed in HL-60 cells (Supplementary Fig. S1C), a promyelocytic leukemia line, which, such as U937 cells, lacks p53 expression because of major deletions in the p53 gene (16) and does not express FLT-ITD (14). In separate studies, sequential administration of MK-8776 for 24 hours before or after HDACIs yielded analogous results in U937 cells, whereas prior HDACI exposure was more effective in p53-wt OCI-AML-3 cells but in no case superior to simultaneous administration (data not shown). In all lines, MK-8776/HDACI coadministration sharply increased caspase-3 (not shown) and -9 cleavage and PARP degradation (Fig. 1C and Supplementary Fig. S1D). Although MK-8776 alone minimally reduced colony formation, it substantially enhanced HDACI inhibitory effects in U937 (Fig. 1D and Supplementary Fig. S1E) and other cell lines (data not shown).

**HDACIs enhance Chk1 inhibition by MK-8776 through downregulation of Chk1**

Effects of MK-8776 ± HDACIs on Chk1 and its downstream signaling cascade were then examined. As reported (8, 11), MK-8776 diminished Chk1 autophosphorylation (Ser296; Fig. 2A and Supplementary Fig. S2A) and downstream Cdc25C phosphorylation (Ser216; Supplementary Fig. S2B). Interestingly, MK-8776 also modestly reduced Chk1 total protein levels, particularly in p53-deficient cells (e.g., U937 and MV-4-11). The pan-HDACIs SBHA or vorinostat, which strikingly increased acetylation of both histone H3 and α-tubulin (Supplementary Fig. S1F) due to class I and II HDAC inhibition, respectively, downregulated Chk1 particularly in p53-wt leukemia cells (e.g., OCI-AML-3 and MOLM-13), a phenomenon consistent with recent reports (24). Notably, these events were clearly greater with combined MK-8776/HDAC1 administration (Fig. 2A and Supplementary Fig. S2A). As shown in Fig. 2B, top and Supplementary Fig. S2C, quantitative PCR (qPCR) analysis showed that HDACI ± MK-8776 also modestly but clearly reduced Chk1 mRNA levels. Moreover, as Chk1 is a known ubiquitination–proteasome system substrate (25), addition of the proteasome inhibitor MG-132 clearly reversed Chk1 protein downregulation and autophosphorylation (Ser296) in MK-8776/HDACI–treated cells (Fig. 2B, bottom and Supplementary Fig. S2D). In contrast, the caspase-independent event. Moreover,
Cdc2/Cdk1 and Cdk2 represent 2 major Chk1 targets in cell-cycle regulation (5). Although MK-8776 or HDACIs alone exhibited little effect, MK-8776/HDACI coadministration markedly reduced inhibitory Tyr14/Thr15 phosphorylations of Cdk2, and to a much more limited extent, of cdc2/Cdk1 in all lines, associated with Cdc25A accumulation and diminished Cdc25C Ser216 phosphorylation (Supplementary Fig. S2A).

Genetic disruption of Chk1 function induces S-phase arrest and sensitizes cells to HDACIs

To validate the functional role of Chk1 in MK-8776 and HDACI lethality, U937 cells were transfected with wt Chk1 (Chk1WT) or a kinase-dead Chk1 mutant (Chk1KD, D130A; Fig. 2C, inset). Although Chk1KD did not modify MK-8776 activity, it significantly increased HDACI and MK-8776/HDACI lethality (Fig. 2C, right; Chk1KD vs. Chk1WT, 36.5% vs. 23.2% or 65.3% vs. 49.4% for vorinostat alone or in combination with MK-8776; P = 0.0019 and 0.0003, respectively; Supplementary Fig. S3A). Chk1KD induced S-phase arrest (Fig. 2C, left and Supplementary Fig. S3B), analogous to MK-8776 effects in Chk1WT control. Interestingly, while HDACIs induced G2–M arrest in both Chk1WT and Chk1KD cells, HDACI treatment induced a further increase in the S-phase population only in Chk1KD cells (P = 0.0373 vs. untreated control), accompanied by a clear increase in the subdiploid fraction (Fig. 2C, left and Supplementary Fig. S3B). Moreover, U937 cells transfected with a construct encoding a Chk1 shRNA displayed a marked reduction in Chk1 protein (Fig. 2D, top), and exhibited, albeit to a lesser extent, increased sensitivity to HDACIs (Fig. 2D, bottom and Supplementary Fig. S3C).

MK-8776/HDACI coadministration disrupts the intra-S-phase checkpoint and DNA replication

Cell-cycle analysis conducted in cells exposed to MK-8776 ± HDACIs before pronounced apoptosis revealed that MK-8776 induced S-phase arrest in U937 (P = 0.002...
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Figure 2. HDACIs enhance Chk1 inhibition by MK-8776, whereas genetic disruption of Chk1 increases S-phase population and sensitizes cells to HDACIs. A, cells were exposed to MK-8776 \( \pm \) 1.5 \( \mu \)mol/L vorinostat for 24 hours, after which total and autophosphorylated (Ser296) Chk1 were monitored. B, OCI-AML-3 cells were incubated with 500 \( \mu \)mol/L MK-8776 \( \pm \) 1.5 \( \mu \)mol/L vorinostat for 16 hours, after which Chk1 mRNA levels were determined by qPCR (top). OCI-AML-3 cells were incubated with 500 \( \mu \)mol/L MK-8776 \( \pm \) 1.5 \( \mu \)mol/L vorinostat in the presence or absence of 300 \( \mu \)mol/L MG-132 for 16 hours, after which total Chk1 levels were monitored by immunoblotting analysis (bottom). C, U937 cells were stably transfected with GFP-Chk1\textsuperscript{WT} or GFP-Chk1\textsuperscript{KD} (inset) and then exposed to 500 \( \mu \)mol/L MK-8776 \( \pm \) 1.5 \( \mu \)mol/L vorinostat for 24 hours, after which 7-AAD\textsuperscript{+} cells was determined in the GFP-positive population (right; \( \text{n.s.}, P = 0.5374; **, P = 0.0019; $$$, P = 0.0003 \) ), and cells cycle analyzed (left). D, Chk1 was knocked down by shRNA (shChk1, 2 clones designated F8 and D2; shNC, a scrambled sequence negative control) in U937 cells. Expression of Chk1 was monitored. Alternatively, cell-cycle analysis was conducted.

VS. untreated control, AML-3 (\( P = 0.0143 \)), MOLM-13 cells (\( P = 0.019 \) Fig. 3A and Supplementary Fig. S3D), and MV4-11 cells (data not shown), accompanied by increased EdU incorporation (Fig. 3B and Supplementary Fig. S4A), reflecting DNA synthesis (26), probably due to increased DNA replication initiation/elongation after Chk1 inhibition (7). Notably, HDACI coadministration sharply decreased the S-phase population in all lines (vs. MK-8776 alone; \( P = 0.0007, 0.0163, \) and 0.0025 for U937, OCI-AML-3, and MOLM-13, respectively), accompanied by an increased subdiploid fraction (Fig. 3A and Supplementary Fig. S3D). Moreover, HDACI also substantially diminished the EdU\textsuperscript{+} population in MK-8776–treated cells (Fig. 3B and Supplementary Fig. S4A). Notably, MK-8776/HDACIs markedly downregulated Cdt1 (Fig. 4A and Supplementary Fig. S3E), a protein essential for replication (origin) licensing (27). Of note, this event occurred independent of caspase activation (Fig. 4B). Furthermore, Chk1 knockdown by shRNA mimicked MK-8776 in attenuating HDACI-induced expression of Cdt1 (Fig. 4C and Supplementary Fig. S3F). Together, these results argue that HDACI/MK-8776 coadministration interferes with the intra-S-phase checkpoint and DNA replication in leukemia cells.
Chk1 inhibition in combination with HDACIs promotes DNA damage in association with downregulation of multiple proteins involved in DSB repair via homologous recombination

MK-8776 modestly increased γ-H2A.X expression (Fig. 4D and Supplementary Fig. S4B), a biomarker of DSB (28), as reported before for other Chk1 inhibitors (7). Notably, HDACI coadministration strikingly promoted MK-8776–mediated γ-H2A.X expression (Fig. 4D and Supplementary Fig. S4B). MK-8776 also markedly induced Chk2 Thr68 phosphorylation, a specific DDR marker in response to DSBs (28), an event enhanced by HDACIs (Fig. 4D and Supplementary Fig. S4B). Moreover, combined treatment markedly diminished CtIP expression (Fig. 4D and Supplementary Fig. S4C), a key protein responsible for resection of DSB ends to generate ssDNA (29) and essential for DSB repair via homologous recombination during S-phase DNA replication (30), via a caspase-independent mechanism (Fig. 4B). It also substantially diminished Ser1423 and Ser1524 phosphorylation of BRCA1 (Fig. 4D and Supplementary Fig. S4C; ref. 31), a CtIP-binding partner (32), also implicated in homologous recombination–mediated DSB repair (32).

Consistently, Chk1 knockdown downregulated CtIP and prevented BRCA1 phosphorylation (Ser1423), accompanied by increased γ-H2A.X expression and Chk2 phosphorylation (Thr68), following HDACI exposure (Fig. 4C and Supplementary Fig. S4D).

p53 knockdown increases p53-wt leukemia cell susceptibility to MK-8776 alone or in combination with HDACIs

As p53-wt cells are generally more resistant than p53-deficient cells to Chk1 inhibitor-based regimens (12, 23), the effects of MK-8776 ± HDACIs on p53 expression and phosphorylation were examined. In p53-wt cells (OCI-AML-3 and MOLM-13), MK-8776 inhibited p53 phosphorylation at Ser20 (Fig. 5A and Supplementary...
Fig. S5A), a Chk1/Chk2-phosphorylating site, which enhances its tetramerization, stability, and activity (33). Interestingly, MK-8776 markedly increased Ser15 phosphorylation, a site phosphorylated by ATM/ATR, which stabilizes p53 by impairing MDM2 binding and preventing proteasomal degradation (34). In accord, MK-8776 clearly increased p53 protein levels. Notably, HDACI coadministration largely abolished p53 phosphorylation on both serine 15 and 20 residues, accompanied by substantial diminishment of p53 protein levels. To assess the functional significance of p53 in these responses, p53 was knocked down by shRNA in OCI-AML3 cells, resulting in downregulation of p53 and its downstream target p21 (Fig. 5B). shChk1 or shNC OCI-AML3 cells were exposed to 1.5 μmol/L vorinostat for 16 hours. After drug treatment, immunoblotting analysis was conducted to monitor expression of the indicated proteins. C, cells were treated as described in A, after which immunoblotting was conducted to monitor DSB (γ-H2A.X expression and Chk2 Thr68 phosphorylation) and expression and/or phosphorylation of the DNA repair proteins CtIP and BRCA1.

The MK-8776/HDACI regimen is active against primary leukemia cells, including primitive CD34+ /CD38− /CD123+ cells, but is relatively less-toxic to normal CD34+ hematopoietic cells

Parallel studies were conducted in primary AML blasts and CD34+ /CD38− /CD123+ subpopulations enriched for primitive leukemia-initiating cells (36). Although treatment with either MK-8776 or HDACIs minimally induced apoptosis, determined by Annexin V–FITC/PI staining, combined exposure substantially increased cell death (e.g., up to 60%) in bone marrow mononuclear cells from patients with AML (Fig. 6A and Supplementary Fig. S6A). Similar results were obtained in cells double-stained with 7-AAD and DiOC6 (reflecting mitochondrial membrane potential, data not shown). In contrast, the identical regimen exerted minimal toxicity toward normal cord blood CD34+ cells (Fig. 6B and Supplementary...
Fig. S6B). Similar results were obtained from 5 additional AML bone marrow (Supplementary Fig. S6C) and 2 cord blood samples (Supplementary Fig. S6D). Interestingly, nontoxic concentrations of MK-8776 (500–700 nmol/L) substantially increased HDACI lethality (Annexin V+) in primitive CD34+/CD38−/CD123+ leukemia progenitor populations (Fig. 6C and Supplementary Fig. S5E). Potentiation of HDACI lethality by MK-8776 was observed in 5 (of 9) AML bone marrow samples from which the primitive CD34+ in 5 (of 9) AML bone marrow samples from which the \( p \)-value was 1.5 \( \mu \)mol/L vorinostat for 16 hours, after which total and phosphorylation (Ser15 and Ser20) of p53 were monitored by immunoblotting. B, p53 was knocked down by shRNA (shChk1, 2 clones designated E4 and D3; shNC, a scrambled negative control) in AML-3 cells. C and D, cells were then treated with 300 nmol/L MK-8776 ± 1.5 \( \mu \)mol/L vorinostat, after which DNA damage (\( \gamma \)-H2A.X; C) or apoptosis (Annexin V−; D) were monitored by immunoblotting and flow cytometry, respectively.

**Discussion**

DNA damage checkpoints protect both normal and transformed cells from genotoxic stress, including that triggered by cytotoxic agents (5). Although HDACIs kill transformed cells (1), attention has recently focused on HDACI-induced DNA damage in neoplastic cells, including leukemia cells (2). Consequently, interference with Chk1 function might also potentiate HDACI lethality in leukemia. The observation that leukemia cells expressing a kinase-dead Chk1 or Chk1 shRNA were significantly more sensitive to HDACIs supports this concept. However, as Chk1 inhibitors induce DNA damage by themselves (2), it is equally plausible that such damage, and resulting lethality, may be amplified by HDACIs. Indeed, the present results show highly synergistic interactions between the Chk1 inhibitor MK-8776 and HDCAIs (e.g., vorinostat) in multiple leukemia cell lines harboring various genetic abnormalities (e.g., wt or deficient/deleted p53, and FLT3-ITD), as well as in primary AML blasts, including primitive leukemic progenitors.

Chk1 has well-established roles in the DDR, particularly in cell-cycle checkpoints (5). To date, the primary rationale for Chk1 inhibitor development has been to enhance genotoxic agent efficacy by abrogating checkpoints (5). Recently, new insights into additional Chk1 roles have emerged, including involvement in origin firing (37), DNA replication, initiation and elongation, and regulation of the intra-S (7) or DNA replication checkpoint in response to replication stress (5). The present results argue that disruption of Chk1 function in S-phase or during DNA replication contributes to MK-8776/HDACI synergism. Specifically, MK-8776 inhibited autophosphorylation (Ser296), which governs basal Chk1 activity (11), accompanied by S-phase accumulation of cells [i.e., intra-S-phase checkpoint (5)], suggesting that basal Chk1 activity is required for S-phase progression (38). Interestingly, MK-8776 also downregulated Chk1, an event potentiated by HDACI coadministration. Of note, HDACIs downregulate Chk1 via both transcriptional and posttranslational mechanisms (3, 24). The observation that the proteasome inhibitor MG-132 partially reversed Chk1 downregulation and attenuation of Ser296 autophosphorylation by MK-8776/HDACIs implicates an
ubiquitin–proteosome–mediated event (25). Moreover, qPCR analysis revealed that Chk1 downregulation by MK-8776/HDACIs might also involve transcriptional repression. In any case, potentiation of MK-8776–mediated Chk1 inhibition or downregulation by HDACIs was accompanied by a striking increase in apoptosis.

It is also possible that MK-8776 arrests cells in S-phase and that cells actively undergoing DNA replication may be particularly susceptible to HDACIs that impair DNA repair (3) and induce DNA damage (2). This concept is supported by the finding that MK-8776 increased EdU incorporation, whereas HDACI coadministration sharply increased apoptosis accompanied by a diminished EdU-positive population. Vorinostat has been shown to produce profound alterations that induce replication-mediated DNA damage (10). Significantly, MK-8776/HDACIs markedly downregulated Cdt1, a replication factor essential for origin licensing (39), a prerequisite for DNA replication initiation (27). It is therefore likely that antileukemic synergism between MK-8776 and HDACIs stems from multiple interactive mechanisms involving disruption of the intra-S-phase checkpoint induced by replication stress.

HDACIs also disrupt the DNA repair machinery by inhibiting or downregulating multiple DNA repair proteins (3). In addition to Chk1 (24), HDACIs also downregulate various DNA repair proteins, particularly those involved in homologous recombination, which preferentially repairs DNA in the S-phase (40), including BRCA1 pathway components (3). Here, MK-8776/HDACI coadministration diminished BRCA1 Ser1524 and Ser1423 phosphorylation, which are critical for proper DSB responses (31). Moreover, MK-8776 or genetic Chk1 disruption also markedly downregulated CtIP, a DNA end resection protein essential for processing DSBs into ssDNA to trigger DSB repair via homologous recombination (29, 30). Interestingly, CtIP acts, through BRCA1 binding (32), as a molecular switch directing cells toward homologous recombination as they enter S-phase (32). Notably, these events were markedly enhanced by coadministration of HDACIs, which induce CtIP acetylation and degradation (41). Studies designed to assess the functional significance of perturbations in BRCA1, CtIP, and Cdt1 in the lethality of this regimen are underway.

Although p53 loss or mutation is relatively uncommon in AML, it strongly influences AML outcome and represents an important adverse prognostic factor (16). Moreover, while p53 loss or mutation occurs infrequently at presentation (e.g., 5%–10%), primarily in patients with 17p monosomy, it is considerably more common in therapy-related AML (t-AML; e.g., 40%–50%; ref. 42). Interestingly, MK-8776 induced marked p53 phosphorylation...
Patients with FLT3-ITD AML have more aggressive disease and a significantly worse prognosis than those with wt disease (44). Notably, MV-4-11 and MOLM-13 cell lines carrying FLT3-ITD (14) were more sensitive to MK-8776 than cell lines bearing wt FLT3 [U937 and OCI-AML3 (14)]. In this context, recent evidence indicates that FLT3-ITD–expressing AML cells exhibit aberrant repair of DSBs via the 2 major DSB repair pathways, e.g., homologous recombination and nonhomologous end joining (44). Collectively, these considerations raise the possibility that the current regimen may be effective against FLT3-ITD leukemia cells, a clinically high-risk AML group. However, given the relatively small number of cell lines examined here, definitive conclusions about the contribution of FLT3 mutations and p53 status to sensitivity to this strategy cannot presently be drawn.

Transformed cells commonly exhibit defective checkpoint responses (5). HDACI selectivity has been attributed to impaired DNA repair in transformed cells (4). The present observations suggest that the MK-8776/HDACI regimen may preferentially target leukemic versus normal hematopoietic cells. Recently, it has been suggested that Chk1 inhibitors may diminish HDACI selectivity in certain normal cell lines, e.g., HPS (45), an SV40-transformed human foreskin fibroblast line (46). However, MK-8776/HDACI toxicity toward normal cord blood CD34+ cells was minimal. In contrast, primary leukemic blasts and CD34+ /CD38− /CD123+ subpopulations enriched for primitive leukemia-initiating cells responsible for AML patients relapse (36) were highly susceptible to the MK-8776/HDACI regimen. In this context, cytogenetically quiescent G0 myeloma cells are highly sensitive to Chk1 inhibitor-based regimens (21). Moreover, the Chk1 inhibitor AZD7762, in combination with genotoxic agents (e.g., ara-C), was shown to target CD34+ /CD38− /CD123+ leukemic progenitors but not normal hematopoietic progenitors (36). The capacity of Chk1 inhibitors to target cancer stem cells (CSC) has also been described in non–small-cell lung (47) and pancreatic cancer cells (48). Interestingly, the Chk1-mediated DDR may be more robust in CSC than nonstem cells (48). In contrast, normal stem cells (e.g., embryonic stem cells, ESC) fail to activate Chk1 in response to DNA replication stress (49). Thus, the present results provide evidence that regimens combining Chk1 with HDACIs may target AML CSC.

In summary, the present findings argue that while Chk1 inhibition potentiates the antileukemic activity of HDACIs, it equally possible that multiple events mediated by HDACIs amplify the lethal consequences of Chk1 disruption (Fig. 7). Significantly, while leukemia cells with deficient-p53 display greater susceptibility to MK-8776 compared with those with wt-p53, MK-8776/HDACI synergism occurs in both p53-wt and deficient cells. Finally, leukemia cells harboring the FLT3-ITD mutation (including those with wt-p53) are sensitive to this regimen, as are primary AML samples, particularly the CD34+/CD38− /CD123+ population enriched for primitive leukemia-initiating cells, but not normal CD34+ hematopoietic cells. Thus, while current approaches using Chk1 inhibitors focus on enhancing conventional genotoxic agent activity, a strategy using Chk1 inhibitors (e.g., MK-8776) to improve the antileukemic activity of HDACIs warrants further attention in AML. Accordingly, studies designed to test this concept are underway.

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

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Conception and design: Y. Dai, S. Chen, S. Grant
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Dai, S. Chen, M. Kmieciak, L. Zhou, X.-Y. Pei, S. Grant
Writing, review, and/or revision of the manuscript: Y. Dai, S. Chen, S. Grant
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