Combining Histone Deacetylase Inhibitor Vorinostat with Aurora Kinase Inhibitors Enhances Lymphoma Cell Killing with Repression of c-Myc, hTERT, and microRNA Levels

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Abstract

MK-0457 and MK-5108 are novel aurora kinase inhibitors (AKi) leading to G2–M cell-cycle arrest. Growth and survival of multiple lymphoma cell lines were studied with either drug alone or in combination with vorinostat, a histone deacetylase inhibitor (HDACi), using MTS and Annexin V assays, followed by molecular studies. Either of the AKi alone at 100 to 500 nmol/L resulted in approximately 50% reduced cell growth and 10% to 40% apoptosis. Addition of vorinostat reactivated proapoptotic genes and enhanced lymphoma cell death. Quantitative PCR and immunoblotting revealed that epigenetic and protein acetylation mechanisms were responsible for this activity. The prosurvival genes Bcl-XL and hTERT were downregulated 5-fold by combination drug treatment, whereas the proapoptotic BAD and BID genes were upregulated 3-fold. The p53 tumor suppressor was stabilized by an increased acetylation in response to vorinostat and a reduced Ser315 phosphorylation in response to aurora kinase A. Vorinostat or trichostatin A decreased MYC mRNA and protein as well as c-Myc-regulated microRNAs. MYC is a critical gene in these responses, as MYC knockdown combined with the expression of the c-Myc antagonist MXD1 raised cell sensitivity to the effects of either AKi. Thus, the HDACi vorinostat leads to both transcriptional and posttranscriptional changes to create a proapoptotic milieu, sensitizing cells to mitosis-specific agents such as AKIs. Cancer Res; 71(11); 3912–20. ©2011 AACR.

Introduction

The aurora kinases are Ser/Thr protein kinases active during late G2 and M phases of the cell cycle. Aurora kinases (AK) A, B, and C regulate key functions during mitosis and thus are logical drug targets for cancer therapies. AK-A is amplified in several tumor types including lymphomas (1), localizes to centrosomes, and is required for spindle body formation. AK-B is present at the midbody of paired sister chromosomes, including the kinetochores. AK-C is expressed predominantly in germ cells and is the least studied member of the family (2). AK-A phosphorylates p53 at Ser315, leading to its ubiquitination by MDM2 and subsequent proteolysis (3). Consequently, depleting cells of AK-A with siRNA leads to p53 stabilization and increased numbers of cells in the G2–M cell-cycle phase (3). Known AK-B substrates include serine 10 of histone 3 (H3-Ser10) and vimentin (4, 5). Here we test the pan-AK inhibitor MK-0457 (previously VX-680; ref. 2) and the AK-A specific inhibitor, MK-5108, alone and in combination with the deacetylase inhibitor vorinostat.

Agents affecting epigenetic targets, such as histone deacetylase inhibitors (HDACi), may enhance the antitumor activity of antimitic agents such as aurora kinase inhibitors (AKi) in several ways. HDACs can upregulate genes involved in DNA damage recognition and response, including those directly involved in cell-cycle control and apoptosis (6, 7). Furthermore, deacetylase inhibitors can lead to apoptosis through acetylation and stabilization of nonhistone proteins such as p53 (8, 9). AK inhibition primarily leads to cell-cycle arrest in the G2–M phase, but not necessarily to cell death. Thus, combining an AKi with an HDACi such as vorinostat (suberoylanilide hydroxamic acid; generic name, vorinostat; trade name, Zolinza; Merck & Co.) may reactivate the proapoptotic capacity of cells and render them more sensitive to apoptosis triggered by cell-cycle inhibition. We show this to be the case, and describe changes in gene-expression levels for MYC (c-Myc), TERT (human telomerase enzymatic reverse transcriptase; hTERT), TP53 (p53), and microRNAs (miRNA) related to lymphogenesis (10, 11), which may contribute to the enhanced sensitivity of cells to AKi in the presence of vorinostat.
Materials and Methods

Cell culture and assays
All cells were obtained from ATCC except the following: L540 cells, from DSMZ; DHL-4 cells, from Dr. Michael Jensen, City of Hope; and KM-H2 cells, from Dr. Markus Müsschen, University of Southern California, all of whom verified cell identities. Cells were grown in RPMI-1640 medium plus 10% FBS and 50 ng/mL Normocin antibiotic (Amaxa/Lonza Biotechnology). Vorinostat, MK-0457, and MK-5108 were from Merck Inc., and were dissolved in dimethyl sulfoxide (DMSO).

Cell growth and survival
MTS assays employed Promega reagents, according to the manufacturer’s protocol. Cells were plated at 5,000 cells/well in triplicate wells of 96-well plates and cultured with the drugs indicated in Figure 1 for 72 hours. MTS reagent was added and light readings at 490 nm were taken 1 to 2 hours later. Raw values were averaged, background absorbencies (medium without cells) subtracted, and resulting values normalized with control cells grown in 0.1% DMSO set to 1 × (100%) growth.

Cell-cycle and apoptosis assays
Log-phase cells were brought to 0.25 × 10⁶/mL and 1 mL aliquots were plated in 12-well plates with drug concentrations as indicated in figures. After 2- and 3-day incubations, cells were centrifuged with cold PBS washes. For cell-cycle analysis, cells were fixed in 70% ethanol and treated with propidium iodide (PI) staining solution: PBS + 0.1% (vol/vol) Triton X-100, 0.2 mg/mL RNase A (DNase-free), and 0.02 mg/mL PI. Cells were incubated for 15 minutes at 37°C and then overnight at 4°C with flow cytometric analysis the next day. For apoptosis determination, cells were assayed by Annexin V-FITC Apoptosis Detection Kit 1 (BD Biosciences) according to manufacturer’s instructions and analyzed by flow cytometry.

RNA isolation, RT, and quantitative PCR
Cells were washed 2 times in cold PBS and cell pellets frozen at −80°C. For mRNA analysis, RNA was extracted with Qiagen EZ-1 reagents according to manufacturer’s recommendations, quantified, and reverse transcribed with Invitrogen SuperScript III reagents, with 2 µg total RNA + 5 ng/µL random hexamers. One-tenth volumes of reverse transcription reactions were analyzed by real-time PCR by Applied Biosystems reagents (ABI) by using either SYBR-Green or Taq-Man 2x Master Mixes. Reactions were run for 40 cycles of 95°C and 60°C alternation, for 15 and 30 seconds, respectively. Quantification was relative to multiple housekeeping genes expressed in lymphatic cells, by the geometric-mean method (12).
For miRNA analysis, cell pellets were extracted with mirVana isolation reagents by Ambion, quantified, and reverse transcribed with miRNA-specific primers and enzyme mix (Ambion or ABI), according to manufacturer’s directions. One-tenth volume of RT product was analyzed with separate, miRNA-specific PCR primer pairs (Ambion or ABI). PCR was with ABI reagents, as described, using the ABI 2x SYBR-Green Master Mix with Ambion primers, and ABI 2x TaqMan Universal PCR Master Mix/No AmpErase-UNG reagents with ABI primers. miRNAs were normalized to miRNA-191 (13) and/or the U6 small nuclear RNA.

Immunoblotting
Western blots were carried out as described (6). Total protein (40 µg) was loaded per lane. All antibodies were from Cell Signaling Technology other than hTERT antibody, which was from Abcam.

G2-M cell-cycle enrichment
Log-phase L540 cells at approximately 0.6 × 10⁶/mL were diluted to 0.25 × 10⁶/mL, grown overnight (~20 hours), and
again brought to $0.25 \times 10^6$/mL. Cells were divided into 4 fractions and drugs added as shown in Figure 4B. Cells were incubated for 24 hours, quickly harvested by 4°C centrifugation, washed once with approximately 500 mL ice-cold PBS, and once with 10 mL of cold-PBS plus protease and phosphatase inhibitors (Sigma). The resulting pellets were lysed and prepared for immunoblotting (6).

**MYC knockdown and MXD1 overexpression**

siRNAs directed against MYC message were sense: 5’-CUGAGACAGAUCAGCAACAACCGdAdA-3’ and antisense: 5’-UUCGGUUUGUUGCUGUCGCAGGA-3’. All nucleotides are ribose form except the 2 underlined at the 3’ end of the sense strand. The negative siRNA was the control, #6201, from Cell Signaling Technology. Overexpression of Myc antagonist Mxd1 was from plasmid pRc/CMV (14, designated “RCMV-Mad”) with empty pRc/CMV-Mxd1, or their controls, were introduced into L540 cells by nucleofection by using reagents and electroporation device by Amaxa/Lonza. Two million cells, concentrated by centrifugation from log-phase cultures, were used per transfection. Electroporation volume was 100 mL L-buffer mixed with supplement and nucleic acids, according to the manufacturer’s instructions, using the X-001 electroporation setting. Final concentration of siRNA during electroporation was 400 nmol/L and amount of plasmid per transfection was 2 μg. Five minutes after electroporation, cells were washed out of cuvettes with prewarmed 0.5 mL antibiotic-free RPMI-1640 with 10% FBS, and added to an additional 1 mL of the same medium. The volume was increased by 5 mL of the same medium the next day. The numbers of viable cells—determined by Trypan blue exclusion and typically being 70% to 75%—were counted and used at 10,000 live cells per well in 96-well plates for MTS experiments, with drug additions begun the day after transfection. Each drug condition was tested in triplicate wells, and MTS reagent added 72 hours after drug addition (96 hours after transfection).

**Statistical methods**

Pairwise comparisons by Dunnett’s test were carried out to compare apoptosis and cell growth/survival between each drug treatment compared with vorinostat (Fig. 1). To evaluate the dose–response relationship of vorinostat to lymphoma cell gene expression, a linear regression was carried out for each gene with dose as the independent variable and individual gene expression as the dependent variable (Fig. 3). T tests were conducted to compare miRNA expression response between the various vorinostat and AKi treatments compared with the DMSO reference (Fig. 5). All significance testing were carried out by SAS V9.2 and all reported P values are 2-sided, using an α-level of 0.05.

**Results**

**Vorinostat and aurora kinase inhibitors curb lymphoma growth singly and together**

We tested single and combined titrations of MK-0457 or MK-5108 and vorinostat in both cell growth and apoptosis...
assays with Hodgkin's lymphoma (HL) cell lines, L540 and KM-H2, and with non–Hodgkin's lymphoma (NHL) cell lines including Daudi, DHL-4, and DHL-6. Figure 1A shows L540 growth inhibition by each drug, as determined by MTS assays. Inhibition was dose dependent and combinations of both drugs inhibited cell growth more than any drug alone at the lower doses. We obtained similar results with the other cell lines tested (Table 1; Supplementary Fig. S1A). Order-of-addition experiments showed no greater effect than with simultaneous addition of drugs (data not shown).

These data allowed us to calculate IC50 and combination index (CI) values. Table 1 shows that for most lymphoma cell lines the IC50s of these drugs were in the submicromolar range (left half of table). The few exceptions were in relative sensitivities to one or the other AKi. For 5 of 6 lines tested—excepting the DHL-6 cells—the IC50 values of MK-0457 were lower than those of MK-5108. We also determined CI values (Table 1, right), showing that combining AKis MK-0457 or MK-5108 with vorinostat had an additive (CI = 0.85–1.1) or frequently synergistic effect (CI = 0.1–0.85). There were no consistent differences in CI values between AKis when combined with vorinostat.

Apoptosis data (Fig. 1B) suggested that the growth inhibition seen in MTS assays was not primarily because of cell-cycle arrest or longer cycling times, but because of time- and dose-dependent increases in apoptosis, as assayed by Annexin V cell labeling. The combination of vorinostat and an AKi was consistently more effective in promoting cell death than any drug alone in L540 cells, with similar data obtained in Daudi (Supplementary Fig. S1B), KM-H2, and DHL-4 cells (not shown).

Vorinostat Plus Aurora Kinase Inhibitors for Lymphoma
various treatments are listed in Supplementary Table S1. We
Percentages of cell populations in each cell-cycle phase for
in the G2
MK-0457 (Fig. 2C) produces a large increase in cells arrested
indicative of dead cells, whereas treatment with 100 nmol/L
significantly the 2 drugs together shift a substantial propor-
tion of the L540 cells into the sub-G1 population (Fig. 2D).

The extent of apoptosis with vorinostat plus either AKi
was 2- to 7-fold greater than with either AKi alone, presum-
ably because AK inhibition leads primarily to cell-cycle
arrest rather than cell death. To discriminate between cell-
cycle arrest and death, we carried out cell-cycle analysis,
with representative results for L540 cells shown in Figure 2.
Incubation in 1.5 μmol/L vorinostat (Fig. 2B) enlarges a
modest subpopulation of cells in the sub-G1 region, often
indicative of dead cells, whereas treatment with 100 nmol/L
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in the G2–M phase, and a small increase in the sub-G1 region.
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Table 1. IC50 values and CI for the lymphoma cell lines

<table>
<thead>
<tr>
<th>Cell type</th>
<th>IC50 values (μmol/L) ± SD (n)</th>
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<tr>
<td>Vorinostat</td>
<td>KM-0457</td>
<td>MK-5108</td>
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<td>MK-0457</td>
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<td>L540</td>
<td>0.58 ± 0.10 (5)</td>
<td>0.66 ± 0.09 (4)</td>
<td>1.51 ± 0.21 (4)</td>
<td>0.44 ± 0.150 (4)</td>
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<tr>
<td>KM-H2</td>
<td>0.51 ± 0.05 (6)</td>
<td>0.34 ± 0.10 (3)</td>
<td>2.77 ± 0.39 (4)</td>
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<tr>
<td>Daudi</td>
<td>0.41 ± 0.10 (6)</td>
<td>0.06 ± 0.0 (6)</td>
<td>0.23 ± 0.03 (2)</td>
<td>1.15 (1)</td>
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<tr>
<td>DHL-4</td>
<td>0.63 ± 0.09 (4)</td>
<td>0.20 ± 0.03 (3)</td>
<td>0.33 ± 0.09 (4)</td>
<td>1.00 ± 0.991 (2)</td>
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<tr>
<td>DHL-6</td>
<td>0.47 ± 0.18 (3)</td>
<td>4.5 ± 0.71 (2)</td>
<td>0.51 ± 0.13 (3)</td>
<td>0.74 (1)</td>
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<tr>
<td>L1236</td>
<td>0.60 ± 0.08 (5)</td>
<td>ND</td>
<td>ND</td>
<td>0.36 ± 0.003 (2)</td>
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<tr>
<td>U937</td>
<td>1.50 ± 0.14 (4)</td>
<td>0.44 ± 0.16 (3)</td>
<td>1.27 ± 0.42 (3)</td>
<td>0.65 ± 0.144 (2)</td>
</tr>
</tbody>
</table>

Vorinostat + MK-0457 | 0.59 ± 0.076 (3) | 0.50 ± 0.043 (3) | 1.14 ± 0.960 (2) | 0.93 ± 0.144 (2) |
Vorinostat + MK-5108 | 0.69 ± 0.078 (3) | 0.85 (1) |

The percentage of apoptosis in each condition exceeds that of cells in sub-G1, as Annexin V labels intact cells early in apoptosis as well as further degraded ones.

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tion of the L540 cells into the sub-G1 population (Fig. 2D).
Percentages of cell populations in each cell-cycle phase for
various treatments are listed in Supplementary Table S1. We
obtained similar results with the HL cell line KM-H2 and the
NHL cell line Daudi, a Burkitt’s lymphoma (Supplementary
Fig. S2; Supplementary Table S1). The additivity, or in some
cases, synergy of these 2 drugs is reflected in the enrichment
of sub-G1 phase cells when both drugs are present. Cell size
determination showed most cells treated with MK-0457
were enlarged, whereas those treated additionally with
vorinostat were smaller than control cells (not shown),
consistent with sub-G1 phase dead and/or dying cells. Along
with enlargement, there was evidence of endoreduplication
(8N DNA) in some assays, with small cell populations beyond
the G2–M peak (not shown). The percentage of apoptosis in
each condition exceeds that of cells in sub-G1, as Annexin V
labels intact cells early in apoptosis as well as further
degraded ones.
**Vorinostat brings about changes in lymphoma cell gene expression**

We carried out real-time PCR analysis of drug-treated L540 cells to determine reasons for the drugs’ effects on the cell cycle and apoptosis. AKi treatment had little effect on the expression of the genes we analyzed, in contrast to strong effects seen with HDAC inhibition. Vorinostat led to downregulation of several genes, most notably MYC (Fig. 3A), TERT, and BCL2L1 (Fig. 3B). Vorinostat downregulated another antiapoptotic gene, MCL1, whereas BCL2 levels changed very little (not shown). Because vorinostat downregulated message levels of MYC, we assayed levels of the c-Myc antagonist, MXD1 (originally MAD1) (14), and found it was simultaneously upregulated (Fig. 3A). Such inverse patterns of expression of MYC and MXD1 genes have been seen in multiple cell types studied, often in cells exiting the cell cycle and/or undergoing differentiation (15, 16).

In contrast to downregulation of antiapoptotic Bcl-XL and Mcl-1, vorinostat upregulated the proapoptotic genes BAD, BID, and NOXA (Fig. 3C). Most gene expression changes were apparent within 4 hours of vorinostat addition (Fig. 3A and C) and were still variably present at 24 hours for c-Myc and MXD1 (Supplementary Fig. S2A); TERT and BCL2L1 (Fig. 3B); and NOXA (Supplementary Fig. S2C). However, BAD and BID message level increases were an early event, seen only at the 4-hour time point (Fig. 3C). By 24 hours their expression levels were at baseline or somewhat repressed (Supplementary Fig. S2C).

Immunoblotting experiments confirmed quantitative PCR (qPCR) results and assessed posttranslational changes in L540 cell proteins (Fig. 4). Figure 4A shows vorinostat concentration-dependent increases in acetylation at the histone H3 lysine-9 residue (H3-K9), which were unchanged by addition of MK-0457. Acetylation of p53 seemed less sensitive to vorinostat compared with H3-K9, becoming apparent only at higher concentrations. Acetylation of p53 was also seen in response to MK-0457, with greater response when combined with 3 μmol/L vorinostat (Fig. 4A); acetylation of p53 is known to lead to stabilization (17). MK-0457-mediated increased p53 acetylation was associated with increased protein levels of p53-target p21Waf1/Cip1, and the mRNA levels of p53-target NOXA (Fig. 3C). Although the amount of p21 and p27 proteins increased in response to vorinostat or MK-0457 alone and in response to MK-0457 in combination with the lowest dose of vorinostat, the levels of these proteins subsequently decreased in response to MK-0457 in combination with the highest dose of vorinostat. This is in agreement with other studies showing that downregulation of p21 or p27 makes cells more prone to apoptosis (6, 18) and is also consistent with accumulation of cells in sub-G1 (Fig. 2). The Western blot data in Figure 4A confirmed at the protein level the downregulation of c-Myc as gene and FOXO3A genes detected by qPCR (Fig. 3A and data not shown). Similarly, Bcl-XL and Mcl-1 protein levels were also reduced (Fig. 4A).

Cell-cycle block experiments using the microtubule poison nocodazole allowed us to enrich for protein isoforms transiently present during the G2–M phase that are difficult to detect in nonsynchronized cells (Fig. 4B). Utilizing synchronized cell populations we were able to visualize the phosphorylated forms of 3 AK targets by Western blot assay. p53 is normally phosphorylated at Ser315 by AK-A, leading to its association with the ubiquitin ligase MDM2 and proteosome destruction (3). Phosphorylation of p53 at Ser46 is strongly associated with proapoptotic activity of this tumor suppressor (19). Histone H3 is a known substrate for AK-B phosphorylation at serine 10 resulting in dissociation of heterochromatin protein 1 during mitosis (4). To assess the effects of aurora kinase treatment on these substrates, we treated L540 cells with nocodazole, with or without MK-0457, and compared them to cells treated with MK-0457 alone and to control cycling cells. Cell-cycle analyses (not shown) indicated that both MK-0457 and nocodazole blocked cycling; the nocodazole-treated cells with or without MK-0457 were similarly enriched for G2–M phase cells (nocodazole, 42%; MK-0457, 45%; both drugs, 53%). All drug-treated cells also had similar viability (nocodazole, 73%; MK-0457, 75%; both drugs, 79%) All 3 phosphoproteins analyzed were expressed at low levels in cycling cells (Fig. 4B, lane 1) but accumulated at detectable levels in the presence of nocodazole. MK-0457 inhibited the phosphorylation of histone H3 in the presence of nocodazole. p53 phosphorylation at both Ser 315 and Ser46 was also inhibited by MK-0457 in the presence of nocodazole.

**Vorinostat and AKi treatments lead to changes in microRNA levels**

miRNAs are key regulators of cell growth and differentiation by virtue of posttranscriptional inhibition of mRNA stability and/or translation (20, 21). c-Myc transcriptionally activates the miRNA 17–92 cluster (10, 11). Because vorinostat repressed c-Myc message and protein (Figs. 3 and 4), we tested expression levels of 3 members of this miRNA cluster—miR-17.5p, miR-17.3p, and miR-18—in response to vorinostat, the unrelated HDACi trichostatin A (TSA), and MK-0457 in L540 (Fig. 5A) and DHL-4 cells (Supplementary Fig. S5A). Treatment with HDACi resulted in decreases in all 3 miRNAs relative to untreated cells HDACi also induced changes in 3 miRNAs that are not myc-regulated—miR-15b, miR-34a, and miR-155—as shown in Figure 5B and Supplementary Figure S5B for L540 and DHL-4 cells, respectively. The 2 cell types have distinct changes in the expression of these miRNAs, possibly reflecting biological differences between the different lymphoma types involved (HL versus B-cell lymphoma).

**Role of MYC downregulation and MXD1 upregulation by vorinostat AKi combination**

Finally, we sought to determine the importance of HDACi-induced c-myc downregulation in lymphoma cell responses to combined HDAC/AKi inhibition. To model this downregulation in the absence of HDACi, we transfected L540 cells with siRNA directed at MYC mRNA, and/or overexpressed the MYC antagonist, MXD1, followed by titration of either MK-0457 or MK-5108 (Fig. 5C and D). Knockdown of MYC message was difficult in L540 cells, typically reaching a 50% decrease at 48 to 72 hours (not shown). Thus, siRNA-MYC had only a small negative effect on cell survival in response to MK-0457 (Fig. 5C) and a slightly greater effect with MK-5108 (Fig. 5D). MXD1 overexpression (Fig. 5C and D, turquoise line) led to
similar results. However, on combining myc knockdown with MXD1 expression (Fig. 5C and D, green lines) L540 cells clearly became more sensitive to both AKis. The IC₅₀ of MK-0457 (Fig. 5C, blue line) was decreased from approximately 0.5 to 0.1 μmol/L by combined treatment, whereas that of MK-5108 (Fig. 5D, blue line) went from approximately 1.8 μmol/L (by extrapolation) to approximately 0.35 μmol/L. Thus, combining myc knockdown with MXD1 overexpression recapitulates the synergistic effect of combining vorinostat with the AKis, which we postulate is in part due to decreased myc levels after treatment.

Discussion

We have studied the effects of MK-0457 and MK-5108, prototype AKis, in combination with histone deacetylase inhibitor vorinostat. Both drugs inhibit AK-A, and MK-0457 also inhibits AK-B; alone their AKi activity exerts strong negative cell-cycle effects on both HL and NHL cells, but has modest consequences for overall cell growth and survival (Figs. 11 and 2). We hypothesized that AKi-induced arrest of cells in G₁–M phase results in activated intracellular stress signaling pathways, but that in cancer cells this cellular response is blunted by epigenetic silencing of tumor suppressor and proapoptotic genes. Thus, the HDACi vorinostat could potentially exert a synergistic or at least additive effect when combined with AKis. This proves to be the case in lymphoma cells, as also seen in acute and chronic myelogenous leukemia cells when combining vorinostat and MK-0457 (22, 23). Given the similar responses of cells treated with both MK-0457 and MK-5108, we hypothesize that it is inhibition of AK-A that is central to the activity in lymphoma cell lines.

The effects of AK inhibition on gene expression levels are modest, whereas those of vorinostat are extensive. Key effects of HDAC inhibition were downregulation of MYC, TERT, BCL2L1, MCL1, and FOXO3A, and upregulation of cell-cycle inhibitors p21 and p27 and the proapoptotic genes BAD, BID, and NOXA, seen in both qPCR and immunoblot assays. Immunoblotting also showed posttranslational effects of vorinostat and p53, leading to stabilization and increased activity of p53.

Telomerase expression often plays a critical role in cancer cell progression, including hematologic neoplasias (24). The rate-limiting component of the telomerase holoenzyme is the catalytic subunit, hTERT (25). HDACi-induced hTERT regulation has been seen in many cell types (26, 27), typically in the form of TERT derepression (28, 29). This report is the first describing TERT downregulation, with a 25-fold decrease in gene expression following HDAC inhibition in lymphoma cells. The mechanistic reasons for this unique result are unclear and may have interesting cell-type specific implications. The TERT gene is a positive transcriptional target of MYC and is repressed by the Mxd proteins (30, 31). Vorinostat-induced MYC downregulation and MXD1 upregulation in lymphoma cells can thus explain hTERT gene repression. Increased telomerase expression can accompany disease progression, for example, higher expression in chronic myelogenous leukemia (CML) blast crisis patients compared with those in the chronic phase (32). Notably, successful imatinib mesylate treatment of CML reduces telomerase activity (33), whereas high telomerase levels correlate with imatinib resistance (34). These observations suggest HDACi-induced TERT downregulation is a biologically significant event in vorinostat inhibition of lymphoma cell growth.

miRNAs are key regulators of cell growth and differentiation due to messenger RNA downregulation (20, 21). Their differential expression can be used to classify multiple human tumor types, including subtypes of lymphomas (35, 36). We show dose-dependent downregulation of miR-17-5p, miR-17-3p, and miR-18 by vorinostat and TSA in L540 and DHL4 cells. These miRNAs are part of the miR-17–92 miRNA cluster, which is MYC regulated and oncogenic in a Burkitt’s lymphoma mouse model, and is also implicated in other cancers (10, 11, 37). HDACi downregulation of these miRNAs is thus biologically significant and mechanistically plausible, given simultaneous repression of myc levels by HDACi.

Three other non–Myc-regulated miRNAs of significance in lymphomas and other hematologic cancers—miR-15b, miR-34a, and miR-155—displayed responses to HDAC inhibition. miRNAs of the miR-15 and miR-16 family target the mRNA of BCL2 and their upregulation is thus associated with apoptosis (38, 39). We saw dose-dependent downregulation of miR-15b in L540 and DHL-4 cell lines by vorinostat or TSA. miR-34a is a positive transcriptional target of p53 (40) and was strongly upregulated in DHL-4 cells (Supplementary Fig. S5); however, its levels declined in L540 cells with HDACi treatment (Fig. 5). miR-155 is generated from sequences within the non–protein-coding BIC RNA, and both RNAs are upregulated in some HL and diffuse large B-cell lymphoma (DLBCL) samples correlating with the activated B-cell phenotype (41, 42). miR-155 also has antiproliferative and proapoptotic activities in melanoma cells and hematopoietic stem cells (43, 44). We observed increases in miR-155 after HDACi treatment in L540 cells, although it was repressed in DHL-4 cells. Variable behavior of miR-34a and miR-155 may reflect the different lymphoma types represented by L540 and DHL-4 cells. Differential effects on cells, of changes in the miRNA levels after treatment, as opposed to steady state overexpression, may contribute to differences in miR-155 activity between cell types.

We have shown the importance of MYC downregulation in response to vorinostat alone and in the combined response to AKis and HDACis. In another hematopoietic malignancy model, reduced MYC levels are critical for acute myeloid leukemia cell growth arrest by the HDACi valproic acid (45). MYC levels decline in many cell types undergoing differentiation, whereas those of MXD genes increase (15, 16). This counterbalance is consistent with a requirement for both MYC knockdown and MXD1 overexpression combined with AKi treatment, to mimic the synergistic effect of vorinostat combined with an AKi. Deacetylase inhibitors are under intense study in hematologic malignancies, with vorinostat currently Food and Drug Administration–approved for treatment of cutaneous T-cell lymphoma (46). HDACi inhibitory agents have multiple activities in lymphoid cells, ranging from direct antitumor activity to suppression of the activated immune response and cytokine storm (47). We have shown...
the effects of vorinostat on various targets, such as p53, hTERT, Bel-2 family members, c-Myc, and multiple miRNAs. These data strengthen the hypothesis that treatment of tumor cells with deacetylase inhibitors promotes a set of proapoptotic changes at the epigenetic and protein level. This is consistent with data reported in various leukemia types treated with vorinostat (22, 23), in which changes in proapoptotic protein levels led to enhanced activity when combined with AKis. Elucidating the mechanisms by which HDACis sensitize lymphoma cells to other agents should assist in the development of clinical combination trials. Our data suggest that one such trial should include the combination of deacetylase inhibitors with mitotic deregulators such as AKis.

Disclosure of Potential Conflicts of Interest

M.J. Kirschbaum; commercial research grant, Merck. The other authors disclosed no potential conflicts of interest.

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