Synergistic Interaction between Histone Deacetylase and Topoisomerase II Inhibitors Is Mediated through Topoisomerase IIβ

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Abstract

Background: DNA topoisomerase II inhibitors and poisons are among the most efficacious drugs for the treatment of cancer. Sensitivity of cancer cells to the cytotoxic effects of topoisomerase II targeting agents is thought to depend on the expression of the topoisomerase IIα isoform, and drug resistance is often associated with loss or mutation of topoisomerase IIα. Histone deacetylase inhibitors (HDACi) are a novel class of compounds that potentiate the antitumor effects of topoisomerase II – targeting agents.

Methods: The interaction between HDACi and topoisomerase II – targeting agents in cancer cells was evaluated as a function of topoisomerase IIα and topoisomerase IIβ expression. Topoisomerase II isoforms were selectively depleted using small interfering RNA and antisense. Drug-induced formation of cleavable complexes involving topoisomerase IIα and topoisomerase IIβ was evaluated by trapped-in-agarose DNA immunostaining and band depletion assays in the presence and absence of HDACi.

Results: Preexposure to HDACi increased the cytotoxicity of topoisomerase II poisons. This was associated with a down-regulation of topoisomerase IIα expression but had no effects on topoisomerase IIβ. In the setting of HDACi-induced chromatin decondensation and topoisomerase IIα depletion, topoisomerase II poison cytotoxicity was mediated through topoisomerase IIβ cleavable complex formation. The HDACi-induced sensitization was also observed in cells with target-specific resistance to topoisomerase II poisons.

Conclusions: The recruitment of topoisomerase IIβ as a target may overcome primary or emergent drug resistance to topoisomerase II – targeting agents and hence may broaden the applicability of this important class of anticancer agents.

Type II topoisomerases are essential enzymes that regulate the topological state of DNA to facilitate several biological processes. Topoisomerase II exists in two isoforms, topoisomerase IIα and topoisomerase IIβ. Although these enzymes have high sequence homology (1) and a similar mechanism of action (2), they are regulated independently and have been associated with different cellular functions. Topoisomerase IIα is necessary for many biological processes involving double-stranded DNA, including replication, mitosis, and chromatin condensation (3, 4). Topoisomerase IIα is mainly located in the nucleus and peaks in expression during G2–M (5, 6). In contrast, topoisomerase IIβ expression remains stable throughout the cell cycle (7) and may have an important role in transcription (8). Although it was reported that topoisomerase IIβ may not be essential in mitosis, topoisomerase IIα knockdown experiments have shown that topoisomerase IIβ may only partially substitute topoisomerase IIα during chromatin decondensation and cell segregation (9).

Due to their essential roles, topoisomerase II enzymes have been successfully targeted for the treatment of cancer. To maintain genetic integrity during the cleavage and religation of DNA, both topoisomerase isoenzymes form transient bonds with the cleaved DNA called the cleavable complex (10). The stabilization and persistence of these cleavable complexes by topoisomerase II poisons have been associated with DNA damage and cell death (11, 12). Multiple in vitro and in vivo studies have suggested that sensitivity of tumor cells to topoisomerase II poisons depends on access to the targets, as well as target location and expression levels. For clinical use, topoisomerase IIα has been considered the more relevant target (13–17).

A class of drugs that may enhance access to DNA and thereby increase the antitumor activity of topoisomerase II – targeting agents are the histone deacetylase inhibitors (HDACi; refs. 14, 18, 19). We previously reported a sequence-specific and time-dependent potentiation of topoisomerase II – targeting agents by the HDACi, suberoylanilide hydroxamic acid (SAHA), and valproic acid (14, 20). Our data indicated that prolonged...
treatment of cancer cells with an HDACi resulted in the depletion of proteins involved in the maintenance of heterochromatin and led to subsequent chromatin decondensation. Chromatin decondensation increased the access of topoisomerase II–targeting agents to the DNA substrate, which potentiated cell death induced by topoisomerase II–targeting agents. Topoisomerase II drug cytotoxicity is largely dependent upon topoisomerase IIα expression. Here, we investigated the relevance of topoisomerase IIα and topoisomerase IIβ as targets of topoisomerase II poisons in the presence of HDACi. We show that in the setting of HDACi pretreatment, the formation of cleavable complexes involving topoisomerase IIβ rather than topoisomerase IIα leads to topoisomerase II–induced cell death. This may broaden the clinical applicability of topoisomerase II poisons and overcome drug resistance due to acquired depletion and mutations of topoisomerase IIα.

Materials and Methods

Chemicals and antibodies. Trichostatin A, sodium butyrate (NaB), valproic acid, mitoxantrone, and teniposide (VM-26) were purchased from Sigma Chemical Co. (St. Louis, MO). SAHA was provided by Aton Pharma (Merck, Whitehouse Station, NJ). Epirubicin was purchased from Pfizer, Inc. (New York, NY). XK469 was a kind gift from Dr. Fred Hausheer. Cell lines were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L glutamine, and 50 units/mL penicillin, and 50 μg/mL streptomycin (Life Technologies Bethesda Research Laboratories, Carlsbad, CA). Cells were incubated in a humidified atmosphere with 5% CO2 at 37°C.

Cell lines. SKBr-3, MCF-7, KM12C, A375, BT-474, and MDA-MB-361 cells were purchased from the American Type Culture Collection (Manassas, VA). Doxorubicin-resistant MCF-7 cells (MCF:Dox) were a kind gift from Dr. Fred Hausherr. Cell lines were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L glutamine, and 50 units/mL penicillin, and 50 μg/mL streptomycin (Life Technologies Bethesda Research Laboratories, Carlsbad, CA). Cells were incubated in a humidified atmosphere with 5% CO2 at 37°C.

Cytotoxicity assays. Topoisomerase II poison cytotoxicity was evaluated in the presence of HDACi by apoptotic and clonogenic assays.

Apoptosis was scored by the presence of nuclear chromatin condensation and DNA fragmentation and evaluated with fluorescence microscopy using bis-benzimide staining. Briefly, cells were treated with an HDACi for 48 hours, after which the media was removed and replaced with media containing the indicated concentrations of a topoisomerase II poison. After 4 hours, the topoisomerase II poison was removed, and the cells were cultured for an additional 48 hours. Cells were harvested using a Cell Scraper (Fisher, Hampton, NH), fixed in 4% paraformaldehyde for 10 minutes at room temperature, and washed with PBS. Cell nuclei were stained with 0.5 μg/mL of bis-benzimide trihydrochloride (Hoechst #33258, Molecular Probes, Eugene, OR). Two hundred cells were counted for each experiment and evaluated for apoptotic scores (apoptotic nuclei/all nuclei × 100). Each experiment was repeated thrice, and the SE was calculated.

For clonogenic assays, cells were plated on six-well dishes at a density of 150 per well and allowed to adhere for 24 hours. For drug combination studies, cells were incubated with medium containing 0.5 mmol/L valproic acid. At this concentration of valproic acid, valproic acid had no discernable effects on growth or apoptosis. After a 48-hour treatment with valproic acid, the medium was removed and replaced with medium containing 0, 1, 10, 25, 50, or 100 nmol/L epirubicin for 4 hours. Epirubicin was then removed, and colonies were allowed to grow for 14 to 21 days to a maximal size of 2 to 3 mm in diameter, stained with 2% crystal violet in methanol, and counted. Colonies were included in the assessment if measuring at least 0.2 mm. For single-drug samples and untreated controls, saline was used in lieu of valproic acid or epirubicin or both. All experiments were done in duplicates and repeated at least thrice. The concentration of epirubicin required for IC50 was determined using the CalcuSyn software as described previously (14). Synergistic effects versus additive effects were also determined by the CalcuSyn program. The fractional inhibition of valproic acid was depicted as the number of observed cells (treatment group) divided by the number of expected cells (untreated control) with a range of 0 to 1.

Microarray. Expression levels of topoisomerase IIα mRNA were evaluated by microarray analysis using Affymetrix Genechips (Affymetrix, Santa Clara, CA) by standard protocols (Moffitt, Molecular Biology Core). Hybridization to Affymetrix chips was analyzed using Affymetrix Microarray Suite 5.0 software. Signal intensity was scaled to an average intensity of 500 before comparison analysis. The MAS 5.0 software uses a statistical algorithm to assess changes in mRNA abundance in a direct comparison between two samples (Statistical algorithms description document. http://www.affymetrix.com/support/technical/whitepapers.html). This analysis is based on the behavior of 16 different oligonucleotide probes designed to detect the same gene. Using the programmed default values, probe sets that yielded a change of P < 0.04 were identified as changed (increased or decreased), and those that yielded a P between 0.04 and 0.06 were identified as marginally changed.

Western blot analysis. Samples were prepared using SDS lysis buffer [2% SDS, 10% glycerol, 0.06 mol/L Tris (pH 6.8)] and evaluated for protein concentration using the bicinchoninic acid method (Pierce, Rockford, IL). Proteins (50 μg) were separated on 8% SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked in tris-buffered saline containing 0.05% Tween 20 (TBST), 5% nonfat milk and incubated with primary antibody in TBST, 5% nonfat milk, overnight at 4°C. Membranes were washed thrice for 10 minutes with TBST and incubated with the appropriate secondary antibody in TBST, 5% nonfat milk for 90 minutes at room temperature. Antibody binding was visualized by chemiluminescence on autoradiography film. Relative expression of proteins was determined by densitometry analysis of at least two scanned autoradiography films from independent experiments using Photoshop software.

Immunofluorescence. MCF-7 cells were treated with 2 mmol/L valproic acid for 48 hours. Cells were harvested by trypsinization, washed in PBS, and adhered to glass slide using Cytospin Funnels (Shandon, Pittsburgh, PA) at a density of 1 × 106/mL. Slides were blocked with 2% bovine serum albumin (BSA) in PBS for 1 hour and probed with antibodies specific for topoisomerase IIα, KiS1 (monoclonal, 1:50), and acetylated histone H3 (polyclonal, 1:100) in 1% BSA/PBS for 1 hour at room temperature in a humidified chamber.

Slides were washed in PBS and incubated with anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 546 (Molecular Probes) diluted 1:100 in 1% BSA/PBS containing goat serum for 1 hour at room temperature in a humidified chamber. Slides were washed with PBS and coverslipped using Prolong Gold mounting media (Molecular Probes). Images were acquired with confocal microscopy. The square pixel surface area for the respective protein expression was analyzed in at least 50 cells per treatment using Photoshop software. For each measured nucleus, the background staining was measured in the immediate vicinity and subtracted from the average square pixel surface area. Evaluation of secondary antibody staining only served as internal staining control. Levels for the fluorescence detection were set accordingly. Experiments were repeated at least twice.

Antisense/small interfering RNA. Topoisomerase IIα and topoisomerase IIβ protein expression was blocked with antisense oligonucleotides and small interfering RNA (siRNA) duplexes. Antisense: The DNA sequences for the oligonucleotide probes were

Clin Cancer Res 2005;11(23) December 1, 2005 8468 www.aacrjournals.org

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as follows: 5′-CTGCAATGGTGCACCTCCAT-3′ for topoisomerase IIα and 5′-TTTGTAGTGACACGACAGTA-3′ for topoisomerase IIβ as described by Towatari et al. (21). Oligonucleotide (1 μg) was suspended in 100 μL OptiMem (Life Technologies Bethesda Research Laboratories) containing 2 μL Superfect transfection reagent (Qiagen, Valencia, CA). The oligonucleotide suspension was raised to a total volume of 700 μL by the addition of DMEM containing 10% fetal bovine serum and incubated on cell monolayers (1 × 105 cells) for 24 hours. The oligonucleotide mixture was replaced the following day, and the cells were incubated for an additional 4 hours before experimental procedures. Controls included topoisomerase IIα and topoisomerase IIβ sense oligonucleotides as well as incubation of cells with Superfect in the absence of oligonucleotide. siRNA: RNA duplexes for topoisomerase IIα (sense, GGUAUACCGGUUGUUGU-GAAC) and topoisomerase IIβ (sense, GCUUACCUUUGUGU-GACGUU) were purchased from Ambion (Austin, TX). Cells were suspended in 0.1 mL siPort electroporation buffer (3 × 10^6/mL, Ambion), mixed with 1 μg siRNA, and pulsed with 300 V for 0.5 milliseconds. Pulsed cells were incubated at 37°C for 15 minutes before experimentation. The Silencer Negative Control #2 siRNA (Ambion), a nonsense siRNA duplex, was used as a control.

Trapped-in-agarose DNA immunostaining assay. The trapped-in-agarose DNA immunostaining assay (TARDIS) was done as described by Willmore et al. (22) with modifications. Cells (2 × 10⁶ per slide) were imbedded in agarose and lysed [2.5 mol/L NaCl, 100 mmol/L EDTA (pH 10), 10 mmol/L Tris, 1% sarkosyl, 1% Triton X-100] in the presence of protease inhibitors [2 μg/mL pepstatin, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 0.2 mmol/L Na3VO4, and 1 mmol/L phenylmethylsulfonyl fluoride] for 30 minutes. Imbedded cells were washed for 20 minutes with 1 mol/L NaCl in the presence of protease inhibitors and incubated in 2% BSA in PBS for 1 hour. Immunostaining consisted of anti-topoisomerase IIα (454, polyclonal, 1:200) and anti-topoisomerase IIβ (monoclonal, 1:100) in 0.5% BSA for 1 hour at room temperature in a humidified chamber. Slides were washed twice for 10 minutes in 0.5% BSA and incubated with the appropriate secondary antibodies (anti-rabbit 546 and anti-mouse 488; Molecular Probes) at a concentration of 1:200 in 0.5% BSA containing goat serum (1:100) for 1 hour at room temperature. Slides were washed with 0.5% BSA for 10 minutes, dried, and counterstained with 0.5 μg/mL of bisbenzimide trihydrochloride (Hoechst #33258, Molecular Probes). Images were acquired by confocal microscopy and the square pixel surface area of the respective protein was analyzed for a minimum of 50 cells per treatment group. Statistical analysis was done by ANOVA. Experiments were repeated at least twice.

**Band depletion.** The band depletion assay was done as described by Xiao et al. (8). Briefly, 5 × 10⁴ cells were lysed in alkaline lysis solution (200 mmol/L NaOH, 2 mmol/L EDTA), and the lysate was neutralized [neutralization buffer: 1 mol/L HCl, 600 mmol/L Tris (pH 8.0)]. The neutralized lysate was then mixed with 3× SDS sample buffer [150 mmol/L Tris-HCl (pH 6.8), 6 mmol/L EDTA, 45% sucrose, 9% SDS, 10% β-mercaptoethanol], and the lysates were separated on 8% SDS-PAGE gels.

**Results**

**Interactions of topoisomerase II poisons and histone deacetylase inhibitors.** We previously reported an increased sensitivity of tumor cells to topoisomerase II—targeting agents after pretreatment with an HDACi in vitro and in vivo (14, 20). Treatment with HDACi led to dose- and time-dependent histone acetylation and subsequent modulation of genes and proteins essential for the maintenance of heterochromatin. The ensuing chromatin decondensation was associated with an increased binding of topoisomerase II poisons to the DNA substrate and in the presence of topoisomerase II, with increased DNA damage and cell death.

The sensitivity of cancer cells to topoisomerase II—targeting agents has been linked to expression of the topoisomerase IIα isoform, whereas studies on the role of topoisomerase IIβ have been more limited. Our experimental data indicate that preexposure of tumor cells to HDACi potentiates the apoptosis induced by the topoisomerase II poisons epirubicin and mitoxantrone (Fig. 1A). Potentiation was not limited to a certain class of HDACi and was observed with selective (SAHA and trichostatin A) as well as with nonselective (valproic acid) HDACi. Similarly, potentiation occurred with other topoisomerase II—targeting agents, such as doxorubicin, etoposide, and VM-26 (see below; data not shown; and refs. 14, 20).

Densitometry analysis of Western blots (n = 3) evaluating cancer cells derived from different organs, including breast, colon, and melanoma, showed that the examined cell lines with decreased or mutated topoisomerase IIα, required higher concentration of epirubicin for IC₅₀ (Table 1) as assessed by colony-forming assays. As reported by other investigators, these data suggest that not organ specificity but rather expression of the topoisomerase II isoforms was predictive for response (13).

A 48-hour preexposure of these cells to the HDACi valproic acid resulted in a decrease in the IC₅₀ of epirubicin. The concentration of valproic acid used for these experiments affected <10% of cells, resulting in a fractional inhibition of cell growth by valproic acid alone of F₁ < 0.1 compared with untreated cells (Table 1). Isobologram analysis using the CalcuSyn software suggested the interaction between valproic acid and epirubicin was synergistic in SKBr3, MCF-7, A375, BT-474, KM12C, and MCF-Dox cells (data not shown). A decrease in IC₅₀ was not observed in MDA-361 cells with a near depletion of both topoisomerase II isoforms topoisomerase IIα and topoisomerase IIβ. In contrast, in the epirubicin-resistant cell line MCF-Dox, the IC₅₀ of epirubicin was lowered by valproic acid despite a mutation and down-regulation of the...
Topoisomerase IIα protein. Although a potential role of multidrug resistance genes in the potentiation of epirubicin by valproic acid cannot be ruled out in the scope of these experiments, limited microarray experiments do not suggest a change in the expression of MDR and MDR-related proteins (data not shown).

Topoisomerase IIα expression is down-regulated by histone deacetylase inhibitor. It is thought that sensitivity of cancer cells to topoisomerase II–targeting agents is predominantly mediated through topoisomerase IIα (13–17). However, we observed an HDACi-induced sensitization in cells with depleted topoisomerase IIα but not in cells depleted of both topoisomerase II isoforms. The effects of HDACi on the expression of topoisomerase IIα and topoisomerase IIβ were evaluated across sensitive and resistant cell lines. As reported previously, we found that HDACi-induced sensitization of cells to topoisomerase II poisons was maximal after a 24- to 48-hour preexposure to the HDACi (14). Time course evaluating the protein expression of topoisomerase IIα and topoisomerase IIβ showed that after a 48-hour exposure to 2 μmol/L SAHA, rather than an up-regulation, a depletion of topoisomerase IIα protein was observed (Fig. 2A). The depletion of topoisomerase IIα expression was not limited to SAHA but was observed with the short-chain fatty acids valproic acid and NaB as well as the hydroxamic acid trichostatin A (Fig. 2B). The decrease in nuclear protein expression corresponded to a time-dependent reduction in the expression of topoisomerase IIα mRNA (Fig. 2C) and not a change in cellular location (Fig. 2D).

Further define the involvement of topoisomerase IIα in the interaction between HDACi and topoisomerase II poisons, both topoisomerase II isoforms were alternatively depleted by siRNA or antisense and exposed to HDACi before administration of a topoisomerase II poison. We have shown above that treatment with HDACi resulted in a down-regulation of topoisomerase IIα (Fig. 2). Treatment of MCF-7 cells with siRNA resulted in significant depletion of topoisomerase IIα and topoisomerase IIβ protein levels without affecting viability (Fig. 3B; data not shown). Topoisomerase IIα was further depleted with the addition of valproic acid (Fig. 3B). Depletion of topoisomerase IIα by siRNA did not effect the potentiation of epirubicin, mitoxantrone, or VM-26 by valproic acid (Fig. 3C). In contrast, the synergistic activity between HDACi and these topoisomerase II–targeting drugs was abrogated by the depletion of topoisomerase IIβ (Fig. 3C). These findings were mirrored when antisense was used to deplete topoisomerase IIα or topoisomerase IIβ when SAHA was used in lieu of valproic acid (data not shown).

Although the topoisomerase IIα siRNA was exquisitely specific, the topoisomerase IIβ siRNA showed a slight off-target effect on the expression of topoisomerase IIα. This off-target effect unlikely contributed to the abrogation of synergy between valproic acid and topoisomerase II poisons because

Table 1. Percentage expression of topoisomerase IIα and topoisomerase IIβ relative to MCF-7 cells and the IC50 dose (95% confidence intervals) of epirubicin by clonogenic analysis with and without a 48-hour pretreatment with the indicated valproic acid dose in the breast cancer cell lines SKBr-3, MCF-7, BT-474, MDA-361, and MCF:Dox; the colon cancer cell line KM12C; and 0.25 mmol/L valproic acid in the melanoma cell line A375.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>% Expression (95% confidence interval)</th>
<th>IC50, nmol/L (95% confidence interval)</th>
<th>Fractional inhibition by VPA</th>
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<tbody>
<tr>
<td></td>
<td>Topo IIα</td>
<td>Topo IIβ</td>
<td></td>
</tr>
<tr>
<td>SKBr-3</td>
<td>138 (125-151)</td>
<td>99 (95-103)</td>
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</tr>
<tr>
<td>MCF-7</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>A375</td>
<td>52 (49-55)</td>
<td>87 (85-89)</td>
<td></td>
</tr>
<tr>
<td>BT-474</td>
<td>83 (69-97)</td>
<td>38 (16-60)</td>
<td></td>
</tr>
<tr>
<td>KM12C</td>
<td>76 (73-79)</td>
<td>31 (12-50)</td>
<td></td>
</tr>
<tr>
<td>MDA-361</td>
<td>37 (26-48)</td>
<td>9 (2-16)</td>
<td></td>
</tr>
<tr>
<td>MCF-7:Dox</td>
<td>23 (19-27)</td>
<td>64 (56-72)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Epi</td>
<td>22 (19-25)</td>
<td>10 (7-15)</td>
<td></td>
</tr>
<tr>
<td>VPA:Epi</td>
<td>22 (17-27)</td>
<td>7 (4-15)</td>
<td></td>
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<tr>
<td>Dose (mmol/L)</td>
<td>0.5</td>
<td>0.5</td>
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<td>F1</td>
<td>0.0</td>
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NOTE: Effects on cell growth by VPA alone on the respective cell lines were depicted as fractional inhibition (F1). Abbreviations: topo II, topoisomerase II; VPA, valproic acid; Epi, epirubicin.

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the direct depletion of topoisomerase IIα by siRNA in the absence of HDACi effects and the further depletion of topoisomerase IIα by the addition of valproic acid did not sensitize cells to topoisomerase II poisons.

Hence, in the presence of an HDACi, topoisomerase II poison cytotoxicity seems mediated through topoisomerase IIβ. Histone deacetylase inhibitor increases the formation of DNA/topoisomerase IIβ cleavable complexes. Treatment with HDACi result in chromatin decondensation and increased access of topoisomerase II-targeting agents to the DNA substrate (14, 20). Topoisomerase II poisons are thought to confer cytotoxicity through the stabilization of topoisomerase II/DNA cleavable complexes. We evaluated whether the presence of HDACi resulted in the involvement of a specific topoisomerase II isoform in the cleavable complexes stabilized by topoisomerase II poisons.

The cleavable complexes involving topoisomerase IIα or topoisomerase IIβ induced by epirubicin in the presence and absence of a 48-hour pretreatment with 2 mmol/L valproic acid were evaluated by trapped-in-agarose DNA immunostaining assay.

Immunofluorescence analysis of confocal microscopy images showed that at concentrations of epirubicin with minimal effect on the stabilization of cleavable complexes involving topoisomerase IIα (P > 0.05), there was a 3-fold increase in the accumulation of topoisomerase IIβ-containing cleavable complexes stabilized by epirubicin in the presence of valproic acid (Fig. 4A and B). This difference reached statistical
siRNA has no detrimental effects on cell cycle progression. In adult mammalian cells, disruption of topoisomerase II embryogenesis (30), the loss of topoisomerase II transgenic studies have shown that although topoisomerase for the endogenous topoisomerase II enzyme (29). Murine cells has made these enzymes attractive targets for cancer therapy. Topoisomerase II exists in two isoforms, topoisomerase IIα and topoisomerase IIβ. The depletion of topoisomerase IIα band, whereas no effects were seen with valproic acid alone. In cells preexposed to 2 mmol/L valproic acid, VM-26 induced an 81% reduction in the topoisomerase IIα band. The band pattern of topoisomerase IIα and topoisomerase IIβ was evaluated in MCF-7 cells exposed to VM-26 and the HDACi valproic acid (Fig. 4G). Band depletion assays allow detection of topoisomerase IIα and topoisomerase IIβ complexed with DNA. The topoisomerase enzymes that are covalently bound to DNA (cleavable complexes) are prohibited from entering the acrylamide gel resulting in a band depletion, whereas topoisomerase II enzymes not bound to DNA readily enter the gel. Exposure of cells to VM-26 resulted in a 52% depletion of topoisomerase IIβ band, whereas no effects were seen with valproic acid alone. In cells preexposed to 2 mmol/L valproic acid, VM-26 induced an 81% reduction in the topoisomerase IIβ band, suggesting an increase in cleavable complex formation Fig. 4C (bottom).

VM-26 induced a 51% depletion in the topoisomerase IIα band. There was a 46% decrease in the topoisomerase IIα band after treatment with valproic acid alone. In cells treated with valproic acid before VM-26, there was a 92% depletion of the topoisomerase IIα band (Fig. 4C, top). However, as discussed above, treatment with valproic acid resulted in a down-regulation of topoisomerase IIα protein expression (Fig. 2A and B and Fig. 3B). The further reduction in the topoisomerase IIα band by VM-26 in the presence of valproic acid resulted from the additive effects of the HDACi-induced down-regulation of the protein and the loss of topoisomerase IIα band due to cleavable complex formation induced by VM-26. This is further supported by the trapped-in-agarose DNA immunostaining assay showing no increase in the topoisomerase IIα–cleavable complexes induced by valproic acid alone. These findings suggest that treatment of cell with valproic acid enhanced the formation of cleavable complexes involving topoisomerase IIβ but not topoisomerase IIα in the presence of VM-26.

**Discussion**

Topoisomerase II is an essential enzyme for cell survival, and the increased expression of topoisomerase II in many cancer cells has made these enzymes attractive targets for cancer therapy. Topoisomerase II exists in two isoforms, topoisomerase IIα and topoisomerase IIβ, with partial functional redundancy (9, 25–28). In yeast, either isoform may substitute for the endogenous topoisomerase II enzyme (29). Murine transgenic studies have shown that although topoisomerase IIα may compensate for a loss of topoisomerase IIβ during embryogenesis (30), the loss of topoisomerase IIα leads to embryonic death (31). However, topoisomerase IIβ seems essential for neuromuscular development after birth (30). In adult mammalian cells, disruption of topoisomerase IIβ by siRNA has no detrimental effects on cell cycle progression. In addition, topoisomerase IIβ may compensate, albeit only partially, for topoisomerase IIα during chromatin condensation and cell segregation (9, 32). Despite similar catalytic activities of the topoisomerase II isoenzymes, cell survival has been correlated with the expression of topoisomerase IIα (12, 33). The role of topoisomerase II as drug targets for topoisomerase II poisons has been evaluated in several studies; and it has been suggested that topoisomerase IIα is the relevant target of topoisomerase II poisons. Disruption or mutation of topoisomerase IIα was associated with drug resistance (34–36). In particular, there are several reports suggesting topoisomerase IIα to be an important target for etoposide, the drug used predominantly for this study; however, much less is known about the role of topoisomerase IIβ as a drug target for etoposide (17). There currently are several drugs approved for clinical use that predominantly target topoisomerase IIα, whereas the clinical utility of topoisomerase IIβ–specific drugs is still under investigation (37).

Here, we provide evidence that HDACi render topoisomerase IIα a relevant target and effector substrate for topoisomerase II poisons. We have shown that preexposure of tumor cells to HDACi led to histone acetylation and down-regulation of proteins essential for the maintenance of heterochromatin (20). The ensuing chromatin decondensation was associated with increased binding of topoisomerase II poisons to the DNA substrate (14, 20). The kinetics of the HDACi-induced chromatin decondensation suggested that a 48-hour preexposure was optimal for synergistic activity.

We now show that exposure of tumor cells to HDACi significantly reduced the concentrations of topoisomerase II poisons required for growth inhibition, even in cells manipulated for target-specific resistance to topoisomerase II–targeting agents. Sensitization was not tissue specific but was observed in breast, colon, leukemia, melanoma, and sarcoma cell lines. We observed a time-dependent reduction in the expression of topoisomerase IIα mRNA and protein in the presence of HDACi that was maximal at 48 hours. In contrast, the effects of HDACi on topoisomerase IIβ were minimal. Modulation of the topoisomerase II isoforms with siRNA and antisense showed that depletion of topoisomerase IIβ abrogated the HDACi-induced potentiation of topoisomerase II poisons, whereas the depletion of topoisomerase IIα did not affect this synergistic interaction. Furthermore, topoisomerase II poison cytotoxicity in the presence of HDACi was associated with an accumulation of cleavable complexes involving topoisomerase IIβ. The depletion of topoisomerase IIα and increase in topoisomerase IIβ–containing cleavable complexes provide a strong argument for a more prominent role of topoisomerase IIβ in the synergistic interaction between HDACi and topoisomerase II–targeting agents.

Combinations of HDACi and topoisomerase II–targeting agents have been reported by several investigators. Although Johnson et al. observed a decrease in sensitivity of leukemia cells to etoposide after preexposure to an HDACi (38), Tsai et al. observed a sensitization of leukemia cells to etoposide (18). This discrepancy is most likely explained by differences in HDACi preexposure and drug concentration. Where Johnson et al. preexposed HL-60 cells to 100 nmol/L trichostatin A for 0.5 hours, Tsai et al. used 400 nmol/L trichostatin A for 4 hours. Since then, other groups have reported increased
sensitivity of several tumor cell lines to topoisomerase II targeting agents after preexposure to an HDACi (14, 19, 20, 39, 40). Synergy was dependent on sequence and timing of drug administration and associated with HDACi-induced chromatin decondensation (14, 19).

Although topoisomerase IIα has been proposed as the effector molecule for cytotoxicity for topoisomerase II poison, the specific roles of topoisomerase IIα and topoisomerase IIβ in the interaction between HDACi and topoisomerase II poisons have not been defined.

Fraser et al. reported an initial activation of the topoisomerase IIα gene promoter with a transient up-regulation of topoisomerase IIα protein expression in leukemia cells treated with the HDACi NaB for 18 to 24 hours. This promoter activation was reversed with longer HDACi exposure, resulting in topoisomerase IIα promoter activity repression (41). In contrast, Kim et al. found no change in the expression or activity of topoisomerase IIα in glioblastoma cells exposed to the HDACi SAHA (19); however, the exposure times may have differed. We observed an HDACi-induced depletion of
topoisomerase IIα mRNA and protein. Maximal effects only occurred after a prolonged exposure to the HDACi (48 hours). Topoisomerase IIα depletion was not limited to a specific HDACi class but was observed with both short-chain fatty acid and hydroxamic acid HDACi. In part, the differences between the findings presented in this report and those presented by others may be explained by differences in the duration of HDACi exposure. More importantly, these findings may also suggest a limited role of topoisomerase IIα in the observed synergy between an HDACi and a topoisomerase II poison. This is further supported by our findings showing that near-complete depletion of topoisomerase IIα by siRNA or antisense in the presence of an HDACi did not inhibit the potentiation of topoisomerase II poisons by HDACi. The HDACi-induced depletion of topoisomerase IIα mRNA and protein expression was maximal at 48 hours, which correlated to maximal HDACi-induced chromatin decondensation and the optimal timing of synergy between HDACi and topoisomerase II–targeting agents. Although topoisomerase IIα was reported to be involved in chromatin condensation (42), a mechanistic link between the topoisomerase IIα depletion and chromatin decondensation cannot be established by the presented data.

More evidence suggesting topoisomerase IIβ as a relevant target in the potentiation of topoisomerase II–targeting agents by HDACi emerges from studies using the topoisomerase IIβ–specific poison XK469. XK469 was shown to induce cleavable complexes in several cell lines in vitro; however, meaningful effects on tumor burden in vivo were not achievable at tolerable doses (43). We found that XK469 by itself only caused minimal apoptosis in breast cancer cells; however, when administered in a sequence-specific combination with HDACi, we observed a potentiation of XK469 induced apoptosis by HDACi. These experiments collectively imply that topoisomerase IIβ is a relevant target in the interaction between HDACi and topoisomerase II–targeting agents. This is further supported by the findings that depletion of topoisomerase IIβ abrogated the synergistic interaction, whereas depletion of topoisomerase IIα did not affect synergy.

Although topoisomerase IIβ may be a target of selected topoisomerase IIα poisons (44–46), most reports suggest that the cytotoxicity for the clinically relevant topoisomerase IIα–targeting agents is determined by topoisomerase IIα levels (47–50). The data presented here show that HDACi potentiates the cytotoxicity of topoisomerase IIα and topoisomerase IIβ–specific topoisomerase II poisons by recruiting topoisomerase IIβ as a target. The results of this study may have several clinical implications. HDACi may lower the concentration of topoisomerase II targeting agents required for activity, thereby limiting adverse effects. Emerging drug resistance due to a loss or mutation of topoisomerase IIα may be circumvented by engaging topoisomerase IIβ as an alternative target. A potentiation of cytotoxicity was not observed in fibroblast exposed to an HDACi before exposure to a topoisomerase II poison (data not shown). Furthermore, preliminary results from an ongoing phase I trial studying a combination of valproic acid and epirubicin did not suggest a potentiation of epirubicin-induced toxicity (Munster et al, ASCO 2005 #A3084). The differential effects of this combination on somatic versus tumor cells may limit tissue-specific toxicities, such as anthracycline-associated cardiotoxicity. In addition, HDACi may increase the clinical utility of β-specific drugs.

Acknowledgments

We thank Dr. Fred Hausherr (Bionumencei Pharmaceuticals Inc., San Antonio, TX) for the MCF-7 cell line and Dr. Robert Snapka (Department of Radiology, Ohio State University, Columbus, OH) for XK469.

References


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