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ARTICLES

Sequence-Specific Potentiation of Topoisomerase II Inhibitors by the Histone Deacetylase Inhibitor Suberoylanilide Hydroxamic Acid

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Abstract Acetylation of histones leads to conformational changes of DNA. We have previously shown that the histone deacetylase (HDAC) inhibitor, suberoylanilide hydroxamic acid (SAHA), induced cell cycle arrest, differentiation, and apoptosis. In addition to their antitumor effects as single agents, HDAC inhibitors may cause conformational changes in the chromatin, rendering the DNA more vulnerable to DNA damaging agents. We examined the effects of SAHA on cell death induced by topo II inhibitors in breast cancer cell lines. Topo II inhibitors stabilize the topo II–DNA complex, resulting in DNA damage. Treatment of cells with SAHA promoted chromatin decondensation associated with increased nuclear concentration and DNA binding of the topo II inhibitor and subsequent potentiation of DNA damage. While SAHA-induced histone hyperacetylation occurred as early as 4 h, chromatin decondensation was most profound at 48 h. SAHA-induced potentiation of topo II inhibitors was sequence-specific. Pre-exposure of cells to SAHA for 48 h was synergistic, whereas shorter pre-exposure periods abrogated synergy and exposure of cells to SAHA after the topo II inhibitor resulted in antagonistic effects. Synergy was not observed in cells with depleted topo II levels. These effects were not limited to specific types of topo II inhibitors. We propose that SAHA significantly potentiates the DNA damage induced by topo II inhibitors; however, synergy is dependent on the sequence of drug administration and the expression of the target. These findings may impact the clinical development of combining HDAC inhibitors with DNA damaging agents. J. Cell. Biochem. 92: 223–237, 2004.

Key words: histone deacetylase inhibitors; topoisomerase inhibitors; synergy; suberoylanilide hydroxamic acid; epirubicin; breast cancer

The topoisomerase II (topo II) inhibitors, doxorubicin (Adriamycin) and 4′ epidoxorubicin (epirubicin, Ellence™), are the backbones of adjuvant therapy for early stage breast cancer. Nonetheless, in many women the tumors are resistant or recur. Modalities that enhance these drugs would greatly impact the treatment of breast cancer. In this study, we show that the histone deacetylase (HDAC) inhibitor, suberoylanilide hydroxamic acid (SAHA), sensitizes breast cancer cells to epirubicin-induced cell death. Sensitization was sequence-specific and required the presence of the target.

Histone acetyltransferases (HATs) and HDACs function to modulate gene expression by the addition or removal of acetyl groups [Grunstein, 1997]. The addition of acetyl groups to lysine residues of histones by HATs or inhibition of deacetylation by HDACs, results in the weakening of the bond between histones and DNA, increasing DNA accessibility and gene transcription [Grunstein, 1997]. There are several HDAC inhibitors under development,

Abbreviations used: SAHA, suberoylanilide hydroxamic acid; HDAC, histone deacetylase; HAT, histone acetyltransferase; topo II, topoisomerase II; CI, 95% confidence interval; SEM, standard error of the mean; Epi, epirubicin; MDA-361, MDA-MB-361; NaB, sodium butyrate; TSA, trichostatin A; IC50, 50% inhibitory concentration; WB, Western blot; IF, Immunofluorescence; ED, effective dose.

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many demonstrating anticancer activity in vitro and in vivo [Marks et al., 2001]. These include sodium butyrate (NaB), trichostatin A (TSA), oxamflatin, apicidin, depsipeptide, MS-275, and SAHA. The latter three are currently undergoing early clinical testing. In breast cancer cells, SAHA was shown to induce growth arrest, differentiation, and apoptosis. However, we have previously demonstrated that differentiation requires continuous presence of drug and is reversible upon drug withdrawal [Munster et al., 2001]. This may render the clinical use of SAHA more complex. The HDAC inhibitor-induced structural changes of the chromatin may render the DNA more accessible and HDAC inhibitors may therefore be used to potentiate DNA damaging agents such as the topo II inhibitors.

Recent reports have suggested a direct interaction between HDAC1 and topo IIα [Tsai et al., 2000; Johnson et al., 2001]. However, reports of combinations between HDAC and topo II inhibitors are contradictory. Johnson et al. [2001] reported that TSA reduced etoposide (topo II inhibitor)-induced apoptosis. In contrast, other investigators have suggested that TSA or NaB may render leukemia cells hypersensitive to etoposide [Tsai et al., 2000; Kurz et al., 2002]. Opposite effects were found even when using the same HDAC inhibitor and cell model.

In this study, we evaluated the effects of SAHA on topo II inhibitor-induced cell death in breast cancer cells. We found that SAHA acted synergistically with topo II inhibitors enhancing epirubicin-induced apoptosis. However, synergistic activity was only observed when cells were pre-exposed to SAHA for at least 48 h. Pre-exposure to SAHA caused a structural change in the chromatin with an increased association of epirubicin with DNA, augmentation of DNA-strand breaks, and subsequent cell death. Shorter pre-exposure was additive, but synergistic and additive effects were abrogated when SAHA was administered simultaneously or after exposure to epirubicin. These observations may be clinically relevant for the further development of HDAC inhibitors in combination with DNA damaging agents.

MATERIALS AND METHODS

Chemicals and Antibodies

SAHA was kindly provided by Aton Pharma, Inc. Epirubicin and doxorubicin were purchased from Pharmacia, docetaxel from Aventis, paclitaxel from Bristol Myers Squibb, and topotecan from Glaxo Smith Kline. All other reagents were of analytical grade and purchased from standard suppliers. Primary antibodies included 454 for topo IIα (developed by Dr. Dan Sullivan [Sullivan et al., 1993]) and C21 topo I antibody (BD Pharmingen).

Cell Lines

SKBr-3, MCF-7, BT-474, and MDA-MB-361 (MDA-361) were purchased from the American Type Culture Collection. MDA-361 cells have a deletion of one allele of topo IIα [Jarvinen et al., 2000] and are resistant to doxorubicin. MCF-7:Dox were a kind gift from Dr. Fred Hausheer. This cell line was cultured in the presence of doxorubicin and shows an approximate 80% reduction in topo IIα expression. Cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM glutamine, and 50 unit/ml penicillin and 50 μg/ml streptomycin (Gibco BRL). Cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C.

Histone Acetylation

Cells were evaluated for acetylated histones (H3 and H4) in the presence of SAHA by Western blot (WB). Cells were treated with 0.5 μM SAHA for 0, 4, 24, and 48 h and harvested. Whole cell lysate (50 μg) were separated on 15% SDS–PAGE gels and transferred to polyvinyl difluoride (PVDF) membranes. Alternatively, histones were extracted with concentrated sulfuric acid on ice and precipitated with acetone for 1 h at −80°C. Acid extracted protein (0.5 μg) was separated on 15% gels.

Electron Microscopy

Cells were fixed with 4% paraformaldehyde in PBS for 1 h at 37°C and post-fixed with 1% OsO_4. Samples were rinsed with PBS, dehydrated in increasing ethanol concentrations (50%, 70%, 80%, 90%, and 100%), embedded in water permeable LR White resin, and ultrathin sections were collected on nickel grids. Sections were stained with uranyl acetate/lead citrate and examined at 60 kV on a Philips 100M electron microscope. Photographs of the tissue sections were acquired at a magnification of 14,000×.

Cell Cycle Analysis

Cell cycle distribution was assayed according to Nusse et al. [1990] with a Becton Dickinson fluorescence-activated cell sorter and analyzed by Cell Cycle Multi-cycle system (Phoenix Flow System). In brief, adherent cells were harvested in media and resuspended in staining solution containing ethidium bromide (25 μg/ml), sodium citrate (0.1%), NP-40 (0.03%), and RNase (10 μg/ml), followed by isolation of nuclei using citric acid (0.01 M) and sucrose (0.25 M) and analyzed by FACS as described previously [Munster et al., 2001].

Evaluation of Apoptosis

Apoptosis was scored by the presence of nuclear chromatin condensation and DNA fragmentation, and evaluated with fluorescence microscopy using bis-benzimide staining. Briefly, harvested cells were fixed in 4% paraformaldehyde for 10 min at room temperature and washed with PBS. Cell nuclei were stained with 0.5 μg/ml of bis-benzimide trihydrochloride (Hoechst #33258, Molecular Probes). Two hundred cells were counted in three separate fields each per experiment and evaluated for apoptotic scores (apoptotic nuclei/all nuclei $\times$ 100). Each experiment was repeated three times and the standard errors of the mean (SEM) were calculated.

Clonogenic Assays

Cells were plated on 6-well dishes at a density of 150 cells/well and allowed to adhere for 24 h. For pre-exposure experiments, cells were treated with SAHA for 48 h. Medium containing SAHA was removed and the cells were incubated with epirubicin for 4 h. Epirubicin was removed and colonies were allowed to grow for 14–21 days, stained with 2% crystal violet in methanol, and counted. Colonies were included in the assessment if measuring at least 0.2 mm. All experiments were performed in duplicates and repeated at least three times. For post-exposure experiments, cells were exposed to epirubicin for 4 h, after which epirubicin was removed and cells were incubated with SAHA for 48 h. SAHA was replaced with media and colonies were grown for 14–21 days (at least 7 doubling times).

Isobologram Analysis

Fractional inhibition of growth was determined using colony-forming assays and Isobologram analysis with the CalcuSync software program as described by Chou et al. [1994]. This allowed the determination of synergistic, additive, or antagonistic effects from drug combinations. IC50s were calculated as the concentrations required for 50% inhibition of growth.

WB Analysis

Cells were harvested, washed with cold PBS, and lysed in SDS lysis buffer (2% SDS, 10% glycerol, 0.06 M Tris, pH 6.8). Proteins (50 μg) were separated on SDS–PAGE gels and transferred to membranes. Membranes were blocked in tris-buffered saline containing 0.05% Tween-20 (TBST), 5% non-fat milk and incubated with primary antibody in TBST, 5% non-fat milk, overnight at 4°C. Membranes were washed three times for 10 min with TBST and incubated with the appropriate secondary antibody in TBST, 5% non-fat milk for 90 min at room temperature. Antibody binding was visualized by chemiluminescence on autoradiography film.

Nuclear Concentration and DNA Binding of Epirubicin

Cells were exposed to the indicated concentrations of SAHA for 48 h followed by epirubicin for the indicated times or both drugs simultaneously for 4 h. Cells were harvested by trypsinization and washed with PBS. Nuclei were purified as described by Nusse et al. [1990]. Nuclei (150,000) were evaluated for epirubicin binding by fluorometry using the intrinsic fluorescent properties of the drug [Zhou and Chowbay, 2002] (excitation 485 nm, emission 535 nm). Fluorescence intensity was graphed relative to the fluorescence intensity of untreated cells. For DNA binding, cells were lysed with 1% SDS lysis buffer and DNA was precipitated in 0.7 volumes of isopropanol and centrifuged at 10,000*g* for 30 min. DNA pellet was washed with ethanol, air-dried and resuspended in water. Epirubicin fluorescence was measured immediately as above.

Comet Assay

The alkaline comet assay was performed as described by Hazlehurst et al. [2001] to quantify DNA-strand breaks. Due to the denaturing conditions of this assay, both single- and double-strand DNA breaks will be recognized. Briefly, cells were treated with either vehicle or SAHA for 48 h followed by vehicle or epirubicin. Cells were harvested by trypsinization and

washed with cold PBS. Cells (5×10^3) were resuspended in 1% low-melting agarose (BioRad) and layered on frosted glass slides. The slides were submerged in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCL, 1% sodium sarcosinate, 10% DMSO, 1% Triton X-100 containing 25 μg/ml Proteinase K) and incubated for 0.5 h at 4°C. The lysis buffer was removed and the slides were washed four times with alkaline wash buffer (300 mM NaOH and 1 mM EDTA, pH 12.2). Comet tails were induced by electrophoresis for 20 min at 25 V in alkaline wash buffer. Slides were re-equilibrated in TBE buffer (90 mM Tris-hydrochloric acid, 90 mM boric acid, 2 mM EDTA, pH 8.0) for 1 h and stained with Sybr Green (Molecular Probes).

Fifty images per slide were randomly acquired using a fluorescent microscope (Vysis) and analyzed using the ImageQuant software (Molecular Dynamics). The comet moment was calculated using the following equation; comet moment $= \Sigma_{0\text{-}n}$ ((intensity of DNA at distance X) $\times$ (distance))/intensity of total DNA. Experiments were repeated at least three times. Statistical analyses were performed by ANOVA and Student's *t*-test.

RESULTS

SAHA Induces Histone Acetylation and Chromatin Structure Changes

It was shown that exposure to SAHA inhibited deacetylase activity resulting in acetylation of histones, cell cycle arrest, and apoptosis in several breast and other cancer cells [Huang and Pardee, 2000; Huang et al., 2000; Mazumdar et al., 2001; Munster et al., 2001; Vigushin et al., 2001; Butler et al., 2002]. Treatment with SAHA resulted in differentiation of breast cancer cells; however, differentiation was reversible and required the continuous exposure to drug [Munster et al., 2001]. In addition to their antitumor effects, treatment of cells with HDAC inhibitors may result in chromatin decondensation and thereby sensitize cells to cytotoxic agents that interact with DNA. We, therefore, determined whether HDAC inhibition by SAHA was associated with a structural change in the chromatin.

Treatment of the breast cancer cells, MCF-7 with SAHA resulted in acetylation of the histones H3 and H4. As shown in Figure 1A, HDAC inhibition occurred as early as 4 h and was maintained through 48 h. We subsequently assessed potential structural changes in the chromatin by electron microscopy. MCF-7 cells were cultured with 0.5 μM SAHA for 48 h and prepared for electron microscopy as described in detail in Materials and Methods. As seen in Figure 1B, treatment of MCF-7 cells with SAHA resulted in decondensation of the chromatin. Compared to untreated cells, where the chromatin was granular and compact, cells cultured with SAHA showed finely dispersed chromatin. The assessment of the HDAC inhibitor-induced chromatin structure changes over time showed that chromatin decondensation was most profound at 48 h (data not shown).

To determine whether chromatin decondensation was due to SAHA-induced cell cycle arrest, we evaluated the cell cycle distribution in MCF-7 cells after a 48-h exposure to increasing concentrations of SAHA (Fig. 1C). At concentrations SAHA (0.5 μM) sufficient for decondensation of the chromatin, no effects on cell cycle were observed. At concentrations higher than 1 μM, cells accumulated in G1, and at concentrations beyond 5 μM, cells were arrested in G2 [Munster et al., 2001].

Combination Effects and Schedule-Dependence

We speculated that HDAC inhibitor-induced chromatin decondensation increases DNA access to macromolecules thereby sensitizing cells to DNA damaging agents such as topo II inhibitors. Several investigators have reported contradictory findings on the ability of HDAC inhibitors to potentiate or suppress etoposide-induced apoptosis [Tsai et al., 2000; Johnson et al., 2001; Kurz et al., 2002]. We have shown that chromatin changes were most profound 48 h after exposure to SAHA suggesting a potential schedule-dependence when using these drugs in combination. We evaluated the effects of SAHA on epirubicin-induced apoptosis as a function of pre-exposure time to SAHA. MCF-7 cells were pre-exposed to SAHA for 0, 12, 24, and 48 h. Cells were then exposed to SAHA and epirubicin for 4 h and analyzed for apoptosis 24 h later. Prolonged exposure of MCF-7 cells to SAHA alone was associated with a moderate increase in apoptosis (3.5%: 4 h vs. 8.4%: 48 h, $P < 0.005$). As seen in Figure 2A, 48 h pre-exposure to SAHA resulted in a significant enhancement of epirubicin-induced apoptosis compared to SAHA or epirubicin alone

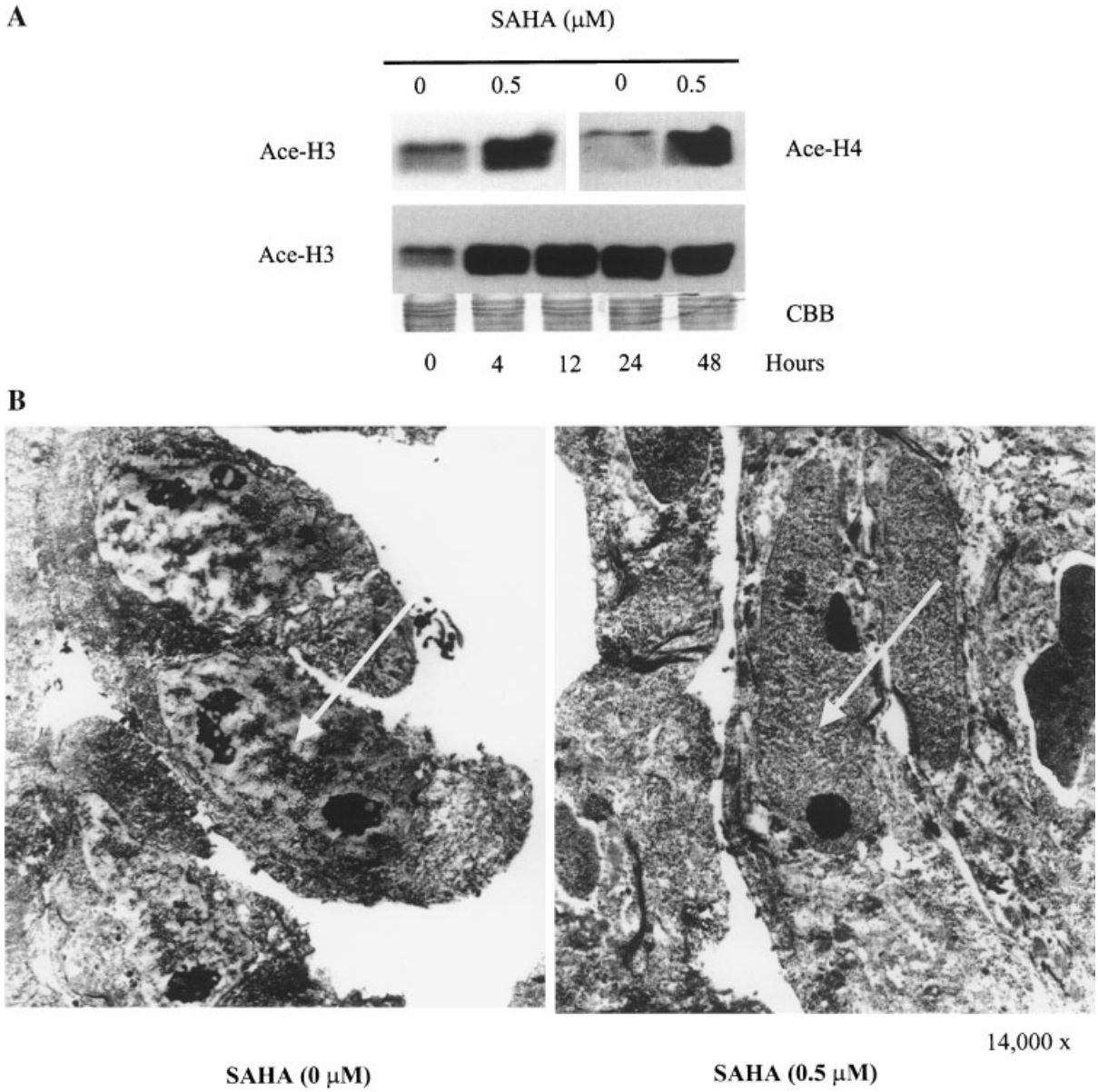


Fig. 1. Effects of SAHA on histone acetylation, chromatin remodeling, and cell cycle. **A**: Whole cell lysates of MCF-7 cells incubated with 0.5 μM SAHA were evaluated for histone H3 and H4 acetylation at 0 and 48 h (**top panel**) and for H3 acetylation at 0, 4, 12, 24, and 48 h (**lower panel**) by WB. Equal protein loading of whole cell lysates was confirmed by Coomassie Brilliant Blue staining (CBB). **B**: Electron micrographs of MCF-7 cells treated with 0.5 μM SAHA for 0 and 48 h and evaluated for chromatin decondensation at 14,000×. Compared to the untreated cells (left), where the chromatin was granular and compact (arrow), cells cultured with SAHA (right) showed finely dispersed chromatin (arrow). **C**: FACS analysis showing the effects of 0, 0.5, 1, and 2 μM SAHA on the cell cycle distribution in MCF-7 cells after 48 h of incubation.

(SAHA → Epi: 26% vs. SAHA: 8.4% vs. Epi: 4.5%, $P < 0.005$). Effects were less pronounced with 24 h pre-exposure (SAHA → Epi: 14.2% vs. SAHA: 9.2% vs. Epi: 4.5%, $P = 0.026$) and abrogated with shorter pre-exposure times ($P > 0.05$ (Fig. 2A)). This pattern of schedule-dependence was also observed in SKBr-3 cells treated with SAHA and epirubicin as well as with SAHA and the non-intercalating topo II poison, etoposide (data not shown).

However, in these experiments, the exposure time to SAHA varied. To circumvent a possible effect of varying exposure times, we evaluated pre- and post-exposure schedules with a defined duration of exposure to SAHA. MCF-7 and SKBr-3 cells were exposed to 0, 0.5, 1, and 2 μM

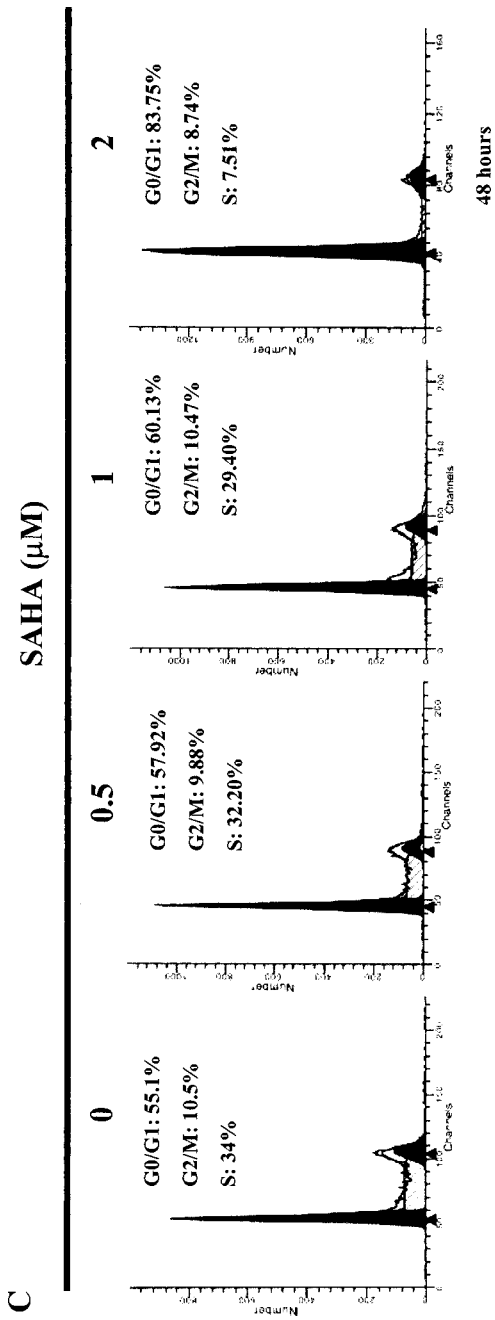


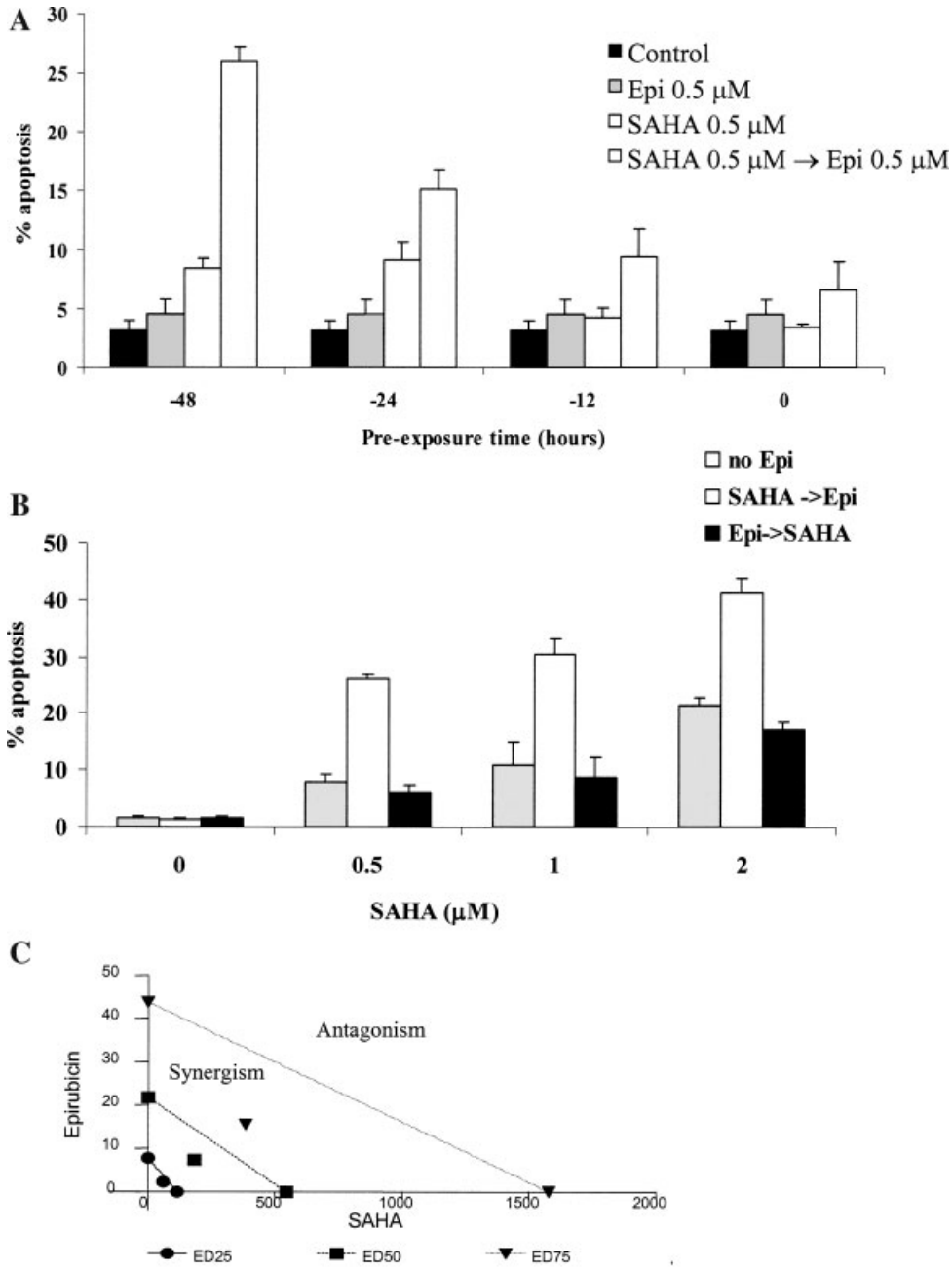
Fig. 1. (*Continued*)

SAHA for 48 h either before or after administration of 0.5 μM epirubicin for 4 h. As shown in Figure 2B, 48 h exposure to SAHA (SAHA → Epi) prior to epirubicin resulted in significant potentiation of epirubicin-induced apoptosis at all examined epirubicin concentrations. Treatment with SAHA for 48 h after epirubicin (Epi → SAHA) was not associated with sensitization to epirubicin but suppressed the epirubicin induced-apoptosis. These findings were confirmed in SKBr-3 cells. Similar results were observed when etoposide was used in lieu of epirubicin (data not shown).

The ability of SAHA to sensitize cells to epirubicin was confirmed by clonogenic assays. MCF-7 and SKBr-3 cells were treated with 0, 0.125, 0.25, 0.5, and 1 μM SAHA for 48 h, SAHA was removed and cells were treated with 0, 5, 10, 20, 50, and 100 nM epirubicin for 4 h (Fig. 2C and data not shown). Colonies were counted after 14–21 days and the fractional inhibition was calculated relative to untreated cells. A decreased survival in the presence of 0.5 μM SAHA was found at all examined concentrations. Synergistic versus additive effects were determined by the use of isobologram analysis as described by Chou et al. (see, Materials and Methods) [Chou et al., 1994]. Isobologram analyses were performed at various concentrations of epirubicin and SAHA with fixed ratios. By isobologram criteria, the combination of SAHA and epirubicin was synergistic when SAHA was pre-exposed to epirubicin for at least 48 h. Combinations of SAHA and epirubicin with shorter pre-exposure or post-exposure were not synergistic (Fig. 2C and data not shown). The IC50 of epirubicin alone was 24.2 nM (CI: 18.3–31.9), for SAHA 544 nM (CI: 342–863). For drug combination, the IC50 for both drugs was lowered to 6.2 nM (CI: 5.8–6.6) for epirubicin and to 155 nM (CI: 145–165) for SAHA (Fig. 2C and Table).

SAHA Enhances Nuclear Concentrations of Epirubicin and Epirubicin-DNA Binding

Topo II inhibitors act by stabilizing topo II:DNA interactions thereby generating cleavable complexes. The formation of cleavable complexes results in DNA-strand breaks. We have shown that exposure of cells to SAHA resulted in a structural change of the chromatin and that pre-exposure but not post-exposure to SAHA sensitized cells to epirubicin-induced apoptosis (Figs. 1 and 2). We then examined



IC 50 (95% CI)	Single Agent	Combination
SAHA (nM)	544 (342-863)	155 (145-165)
Epirubicin (nM)	24.2 (18.3-31.9)	6.2 (5.8-6.6)

Fig. 2. The potentiation of epirubicin cytotoxicity by SAHA was dependent upon schedule of administration. **A**: Potentiation of epirubicin-induced apoptosis as a function of pre-exposure time to SAHA. MCF-7 cells were pre-treated with SAHA (0.5 μM) for 0, 12, 24, or 48 h followed by epirubicin (0.5 μM) for 4 h and assessed for apoptosis 24 h later. Error bars indicate the SEM from three consecutive experiments. **B**: Schedule of SAHA exposure determines potentiation or suppression of epirubicin-induced apoptosis. MCF-7 cells were treated with SAHA (0, 0.5, 1, or 2 μM) either for 48 h before (SAHA → epirubicin) or 48 h directly after (epirubicin → SAHA) exposure to epirubicin (0.5 μM for 4 h) and evaluated for apoptosis. Error bars indicate SEM from three consecutive experiments. **C**: Combinations of SAHA and epirubicin were synergistic by isobologram criteria. Clonogenic assays were performed in MCF-7 cells treated with SAHA for 48 h followed by epirubicin for 4 h at a fixed ratio. The isobologram shows drug combinations at effective doses (ED) 25, 50, and 75. Points falling to the left of the lines are synergistic. The table depicts IC50s of SAHA and epirubicin as single agents and in combination.

whether the HDAC inhibitor-induced sensitization of breast cells was associated with an increase in DNA-epirubicin interaction. We measured the nuclear concentration of epirubicin in cells that were pre-exposed to an HDAC inhibitor. MCF-7 cells were treated with SAHA for 48 h, followed by epirubicin for 4 h, or with both drugs simultaneously for 4 h. Cells were then harvested and nuclei purified. Nuclei (150,000/well) were transferred to 96-well plates and the fluorescence intensity was measured as described in Materials and Methods (Fig. 3). Experiments were repeated three times. The relative fluorescence intensity of untreated cells was 1.0 (95% confidence interval (CI): 0.7–1.3). Exposure to SAHA alone did not result in a significant increase in the relative fluorescence intensity (1.3, CI: 0.8–1.9). Compared to untreated cells, MCF-7 cells exposed to epirubicin exhibited a 7.4-fold (CI: 6.5–8.3) increase in the relative fluorescence intensity. The relative fluorescence intensity was potentiated to 15.1-fold (CI: 14.6–15.5) after exposure to SAHA. In contrast, when MCF-7 cells were treated with SAHA and epirubicin simultaneously for 4 h, there was no significant difference in fluorescence intensity in the absence or presence of SAHA (7.4, CI: 6.5–8.3) vs. 7.7, CI: 7.6–7.8). Epirubicin fluorescence intensity was similar when nuclei were lysed and the chromatin was precipitated and resuspended in water, suggesting a direct DNA-epirubicin interaction rather than non-specific nuclear accumulation (data not shown).

Increased DNA Interaction With Epirubicin Results in Increased DNA Damage

Treatment with the anthracyclines epirubicin and doxorubicin results in DNA-strand breaks [Ross and Bradley, 1981; Zwelling et al., 1982; Capranico et al., 1987]. Anthracycline-resistance has been associated with a decrease in DNA-strand breaks [Capranico et al., 1987, 1989]. As we have shown above, pre-exposure to an HDAC inhibitor was associated with increased nuclear concentration and DNA association of epirubicin, hence we studied whether this resulted in an increase in DNA-strand breaks. We used the alkaline comet assay to compare the amount of epirubicin-induced DNA-strand breaks in the breast cancer cell lines, SKBr-3 and MCF-7, after a 48-h SAHA pre-treatment. The comet moment is a function of both the distance and the amount (measured in pixel density) of DNA that migrates from the center of the head of the comet. As shown in Figure 4A, the tail length, tail intensity, and tail shape differed according to the different treatment conditions. In the cells pre-treated with 0.5 μM SAHA, the epirubicin-induced comet moments were significantly increased (Fig. 4B). Multivariate analyses by ANOVA suggested a statistically significant difference between the combination and the single agent treatments in both SKBr-3 and MCF-7 cells. An increase in the comet moment in the presence of SAHA was also observed with etoposide (data not shown).

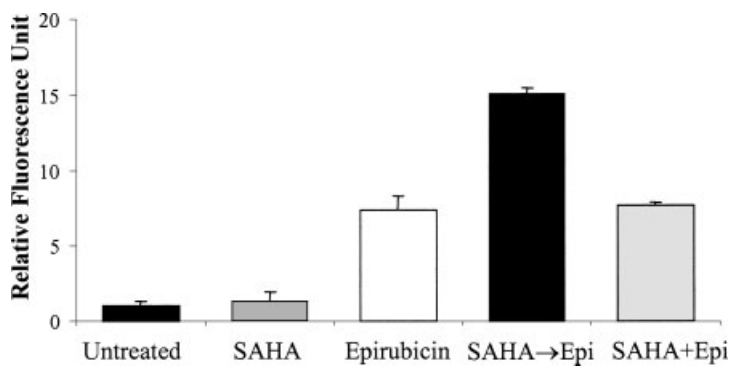


Fig. 3. **A**: Nuclear accumulation of epirubicin. MCF-7 cells were treated with SAHA (0.5 μM) for 48 h followed by epirubicin (1 μM) for 4 h or treated with SAHA and epirubicin together for 4 h and evaluated for nuclear concentrations of epirubicin using the intrinsic fluorescent properties of epirubicin (excitation 485 nm, emission 535 nm). Fluorescence intensity was normalized to untreated cells. Epirubicin fluorescence was increased after pre-exposure to SAHA, but not with concomitant exposure.

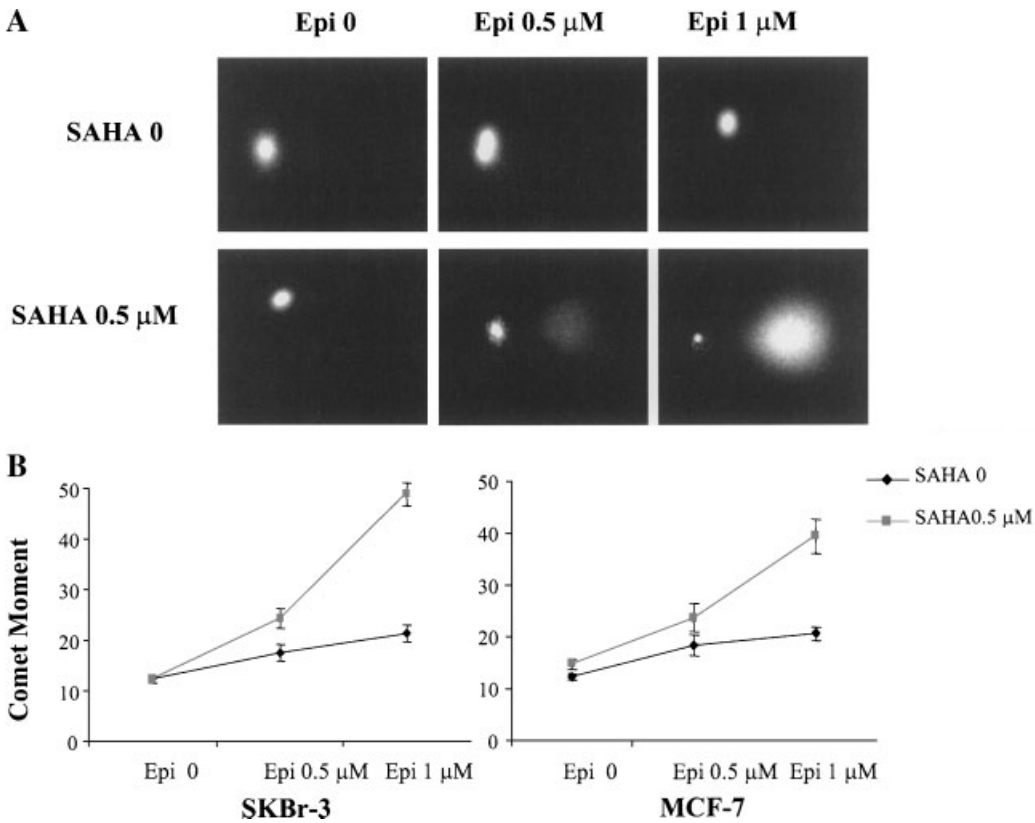


Fig. 4. Assessment of DNA damage induced by epirubicin in the presence or absence of SAHA. The alkaline comet assay was used to compare DNA damage induced by the drug combination in SKBr-3 and MCF-7 cells. Cells were treated with epirubicin (0.5 or 1 μM) for 4 h, in the presence (■) and absence (◆) of a 48-h pre-treatment with SAHA (0.5 μM). **A**: Fluorescence microscopy of MCF-7 cells at 400× magnification showed a difference in the tail intensity, shape, and distance in cells treated with epirubicin and SAHA. **B**: Line graph of comet moment analyses as described in Materials and Methods, error bars depict SEM. Multivariate analysis by ANOVA suggested a significant difference in the comet moments for drug combinations, but not for single agent treatments.

These findings support the hypothesis that pre-treatment of cells with an HDAC inhibitor increases the association of epirubicin with the chromatin and increases the DNA damage induced by epirubicin.

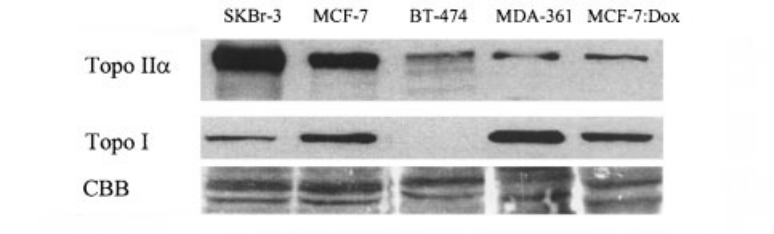
HDAC Inhibitor-Induced Sensitization to Topo II Inhibitors Was Not Observed in Cells With Depleted Topo II

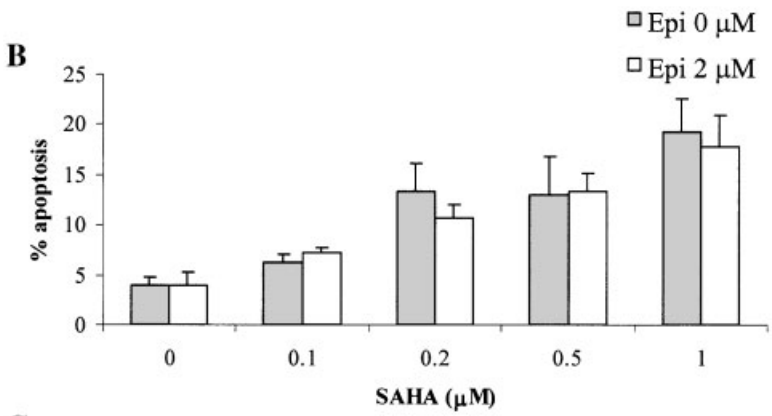
Previous reports have indicated a correlation between sensitivity of cancer cells to topo IIα inhibitors and topo IIα expression levels [Harker et al., 1995; Zhou et al., 2001]. Figure 5A shows the relative topo IIα expression in the breast cancer cell lines, SKBr-3, MCF-7, BT-474, MDA-361 (one allele topo IIα depletion), and MCF-7:Dox (approximately 80% reduction in topo IIα expression) as evaluated by WB and the concentrations of epirubicin required for 50% growth inhibition (IC50) as determined by clonogenic assays. Topo IIα expression was measured from five different cell panels and normalized to 100% for MCF-7 cells (unaltered topo IIα gene expression). The IC50s were calculated from the average of at least three experiments using the CalcuSync software. As shown, sensitivity to epirubicin correlated with topo IIα expression. The IC50s of the examined cell lines were as follows: SKBr-3: 18 nM; MCF-7: 24 nM; BT-474: 38 nM; MDA-361: 132 nM; MCF-7:Dox: 1643 (Fig. 5A). Despite the finding that topo IIα expression in MDA-361 and MCF-7:Dox are similar, the epirubicin IC50s varied significantly. WB analysis of whole cell lysates does not reflect mutations or translocations of topo IIα, which may contribute to resistance.

In order to study the effects of topo IIα in the synergy between SAHA and epirubicin, we evaluated the ability of SAHA to potentiate epirubicin-induced cell death in cells with depleted topo II. Epirubicin-induced apoptosis was evaluated in the epirubicin-resistant breast

A

Cell lines	Topo II α expression(WB) (%)	IC50 (CI) Epirubicin alone (nM)
SKBr-3	138 ± 13	18 (15-25)
MCF-7	100	24 (18-32)
BT-474	83 ± 14	38 (35-40)
MDA-361	37 ± 11	132 (101-174)
MC7-7:Dox	23 ± 4	1643 (980-2753)





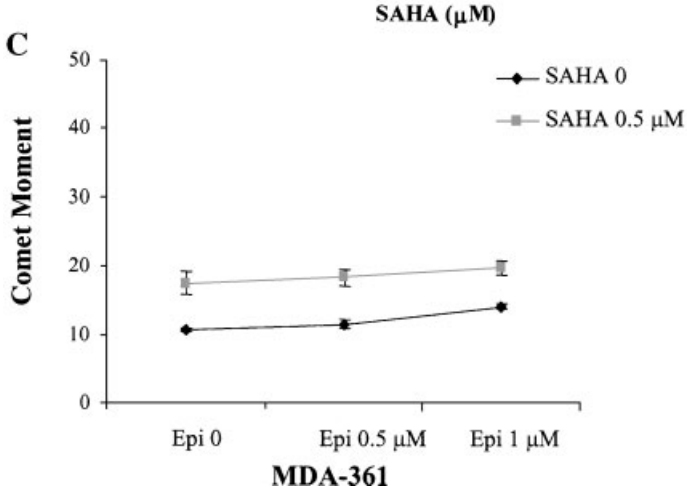


Fig. 5. The relevance of target expression in the sensitivity and SAHA-induced sensitization to epirubicin. **A**: Sensitivity to epirubicin reported as IC50 dose calculated from clonogenic assays in breast cancer cells. Topo IIα expression was measured from five different cell panels and normalized to 100% for MCF-7 cells (unaltered topo IIα gene expression). WB showing the relative expression levels of topo IIα and topo I. Coomassie Brilliant Blue (CBB) control was used for equal protein loading. **B**: Apoptosis induced by epirubicin in cells with depleted topo II expression in the presence and absence of SAHA. MDA-361 cells were treated with 0, 0.1, 0.2, 0.5, or 1 μM SAHA for 48 h followed by 2 μM epirubicin for 4 h. Error bars indicate SEM. **C**: Comet moment in MDA-361 cells incubated with 0, 0.5, or 1 μM epirubicin in the presence and absence of 0.5 μM SAHA pre-treatment. ANOVA analysis indicated no significant differences between combination and single agent treatments. **D**: Changes in the nuclear concentrations of epirubicin after 48 h pre-treatment with SAHA in MCF-7 and MDA-361 cells measured by fluorescent intensity as described in Materials and Methods.

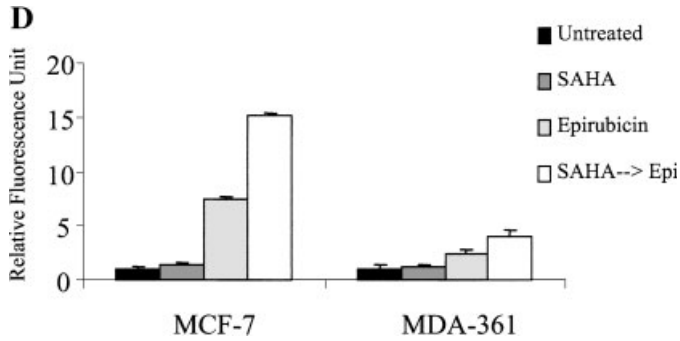


Fig. 5. (*Continued*)

cancer cell line, MDA-361 (IC50: 132 nM epirubicin). These cells have depleted topo II expression (see Fig. 5A and [Jarvinen et al., 1999, 2000]). Cells were cultured with increasing concentrations of SAHA for 48 h prior to exposure to 2 μM epirubicin. In contrast to MCF-7 and SKBr-3 cells (Figs. 2A,B and data not shown), SAHA did not potentiate epirubicin-induced apoptosis in MDA-361 cells (Fig. 5B). Similarly, neither additive nor synergistic effects were observed by clonogenic assays (data not shown). Comet assays were used to evaluate epirubicin-induced DNA damage in the presence and absence of SAHA pre-treatment. Figure 5C shows the comet moments of MDA-361 cells pre-treated with 0.5 μM SAHA followed by 0.5 or 1 μM epirubicin. Multivariate analysis by ANOVA indicated that there were no significant differences between the combination and the single agent treatments. The fluorescence intensity of these cells was measured as described above to determine the nuclear concentrations of epirubicin in the presence and absence of SAHA and compared to MCF-7 cells. As shown in Figure 5D, the relative fluorescence intensity of epirubicin in MDA-361 cells was 2.6 (CI: 2.3–2.9), that increased to 4.4 (CI: 4.2–4.6) in the presence of SAHA, as compared to 7.4 and 15.1 in MCF-7 cells, respectively. While the nuclear concentration of epirubicin in the presence of SAHA showed a relative increase of 70% in MDA-361 the cells, absolute increase was small and did not result in an increase in DNA damage. These experiments do not distinguish whether the DNA:topo II inhibitor interaction is protein-mediated and required the presence of topo IIα. However, fluorescence intensity of epirubicin was not abrogated after DNA precipitation by isopropanol in the presence of 1% SDS suggesting a covalent binding of epirubicin. Compared to MCF-7 cells treated with 1 μM epirubicin alone that had a 7.4-fold increase in the fluorescence intensity, exposure to epirubicin in MDA-361 resulted only in a 2.6-fold increase in fluorescence intensity. This suggests that the levels of topo IIα expression are related to the nuclear accumulation and DNA binding of epirubicin, and that chromatin decondensation is not sufficient to enhance topo II inhibitor-mediated DNA damage in the absence of target expression.

HDAC Inhibitor-Induced Sensitization Is Limited to Topo Inhibitors and Requires the Expression of the Target

To determine if SAHA selectively sensitized cells to topo II inhibitors, we evaluated several classes of antitumor agents in combination. MCF-7 cells were pre-treated with SAHA (0.5 μM) for 48 h, SAHA was removed and cells were exposed to the topo II inhibitors (epirubicin and etoposide), the topo I inhibitor (topotecan), the microtubule inhibitors (docetaxel and paclitaxel), or the platinum derivative (cisplatin). Cells were harvested 24 h later and evaluated for apoptosis. As shown in Figure 6A, synergistic activity was not observed with agents that do not directly interact with the DNA with any sequence of drug administration. In contrast, SAHA potentiated apoptosis induced by the topo I inhibitor, topotecan. Similar to topo II inhibitors, synergy was schedule-dependent.

In Figure 5, we show that despite HDAC inhibitor-induced nuclear accumulation of epirubicin in the topo II depleted cells, MDA-361, sensitization to topo II inhibitors was not conferred. However, these cells express unaltered levels of topo I. To determine the relevance of the target expression, we pretreated MDA-361 cells with SAHA and exposed them to the

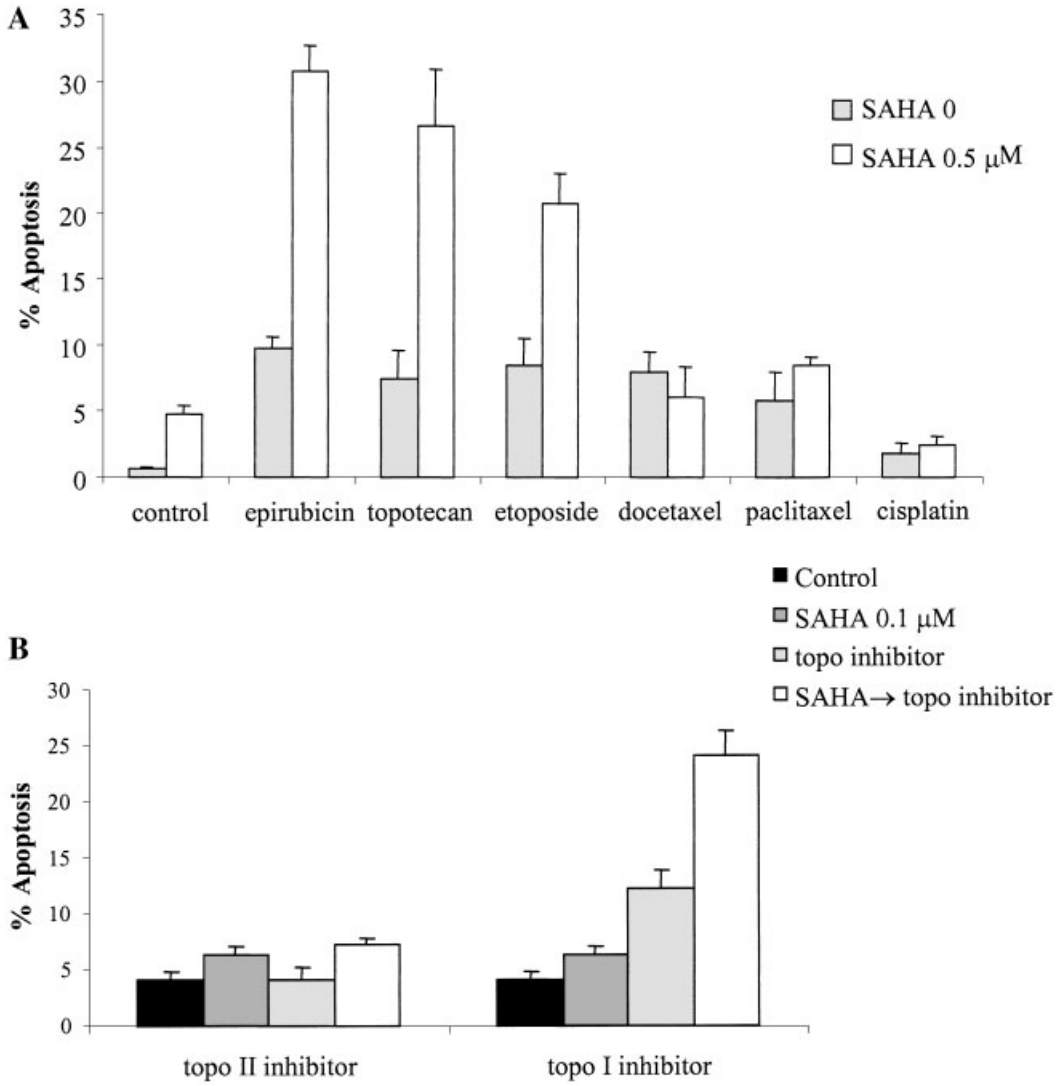


Fig. 6. Assessment of SAHA pre-treatment on apoptosis induced by other cytotoxic agents. **A**: MCF-7 cells were pre-exposed to 0.5 μM SAHA for 48 h followed by a 4-h treatment of 0.5 μM epirubicin, 0.5 μM topotecan, 5 μM etoposide, 0.02 μM docetaxel, 0.02 μM paclitaxel, or 2 μM cisplatin. Apoptosis was evaluated 24 h after drug treatment. **B**: Comparison of SAHA-induced sensitization to a topo II inhibitor versus a topo I inhibitor in MDA-361 cells (topo II depleted). Cells were pre-exposed to 0.1 μM SAHA for 48 h followed by 0.5 μM epirubicin (topo II inhibitor) or 0.5 μM topotecan (topo I inhibitor) for 4 h and evaluated for apoptosis 24 h later. Error bars indicate SEM.

topo I inhibitor, topotecan. As seen in Figure 6B, SAHA potentiated the effects of topotecan in MDA-361 cells, while synergy was not observed with epirubicin. This suggests that synergy may not only depend on enhanced access to the DNA but also the presence of the respective target.

DISCUSSION

The histone deacetylase (HDAC) inhibitors are a class of compounds that may regulate the access of DNA to macromolecules. Several HDAC inhibitors have single agent antitumor activity in vivo and are currently being tested in clinical trials. Treatment of cells with HDAC inhibitors leads to acetylation of histones. Histone acetylation removes the positive charges from the lysine residues of histones and results in weakening of the tight DNA-histone bond. We speculated that exposure of cells to HDAC inhibitors may increase the interaction of DNA with topo II inhibitors resulting in increased lethal DNA damage. Topoisomerases control and regulate the topological states of DNA by cleaving and religating supercoiled DNA [Osheroff, 1989; Sullivan et al., 1989].

Consequently, cells exposed to topo II inhibitors undergo abnormal DNA replication due to the stabilization of cleavable DNA–topo complexes in the nuclear matrix. This leads to DNA damage and subsequent cell death. The most commonly used topo II inhibitors include the anthracyclines (doxorubicin, daunorubicin, and epidoxorubicin (epirubicin)) and epipodophyllotoxin (etoposide). While doxorubicin and epirubicin are among the most active drugs for the treatment of breast cancer, primary or secondary resistance is common and the therapeutic window is narrow. Several investigators studied the antitumor effects of HDAC inhibitors and topo II inhibitors in cancer cells. The reports to date have been contradictory. Both potentiation and suppression of etoposide-induced apoptosis by the HDAC inhibitor, TSA have been reported, even for the same cell models [Tsai et al., 2000; Johnson et al., 2001]. The mode of action of HDAC inhibitors suggests that the potentiation of topo II inhibitors may be schedule-dependent, and that the differential findings by other investigators may be due to different drug treatment schedules used in those studies. In this study, we describe the sensitization of breast cancer cells to topo II inhibitors by the HDAC inhibitor, SAHA.

We found that pre-treatment of breast cancer cells with SAHA for 48 h resulted in a significant potentiation of epirubicin-induced cell death. Determined by isobologram analysis, these effects were synergistic. However, the SAHA-induced sensitization to epirubicin was dependent on treatment schedules. Shorter pre-exposure, concomitant exposure, or exposure to SAHA after treatment with epirubicin resulted in either loss of synergy or antagonistic effects. These findings suggest that the differential observations made by other investigators is likely due to varying HDAC inhibitor pre-exposure times used in those studies. Johnson et al. described that a 30-min pre-exposure of cells to TSA suppressed etoposide-induced apoptosis, whereas Tsai et al. and Kurz et al. reported a potentiation of etoposide-induced apoptosis after a 24–72 h pre-exposure of cells to TSA and NaB, respectively [Tsai et al., 2000; Johnson et al., 2001; Kurz et al., 2002].

We speculated that HDAC inhibition renders the DNA a better target for DNA damaging agents. We showed that treatment of cells with SAHA resulted in a decondensation of the chromatin. While histone acetylation was observed as early as 4 h, we found that the chromatin decondensation was most profound at 48 h. Chromatin decondensation was associated with an increase in the nuclear concentration of epirubicin and an increase in the association of epirubicin with the chromatin. The role of DNA access and sensitivity to topo inhibitors has been investigated by Carrier et al. [1999]. This group reported that gadd45, a p53 responsive protein, modified the accessibility of DNA to topo inhibitors, in particular, in the setting of histone hyperacetylation. However, we found that SAHA increased the access of DNA to epirubicin even in cells with mutated p53. Furthermore, SAHA-induced histone hyperacetylation occurred as early as 4 h while sensitization to epirubicin required a SAHA pre-exposure of at least 24–48 h.

The observed increase in nuclear epirubicin and chromatin-epirubicin associations resulted in a significant increase in DNA damage. DNA damage was measured using the alkaline comet assay. We found that the ratio of the comet moments for epirubicin alone versus SAHA followed by epirubicin did not change over time, suggesting a SAHA-mediated inhibition of repair mechanisms or efflux less likely (data not shown).

Our results suggest that the potentiation of epirubicin-induced DNA damage led to increased cell growth arrest and apoptosis. As described above, synergy between SAHA and epirubicin was schedule-dependent with the most profound effects observed with 48-h pre-exposure to SAHA. In addition, synergy was observed using colony forming assays when cells were pre-exposed, but not post-exposed to SAHA. We previously reported that the differentiation and growth arrest induced by SAHA were reversible and required continuous exposure to drug [Munster et al., 2001]. In the colony forming assays, cells were exposed to SAHA for 48 h followed by a 4-h exposure to epirubicin. The drugs were removed and the colonies counted 14–21 days later. As the cellular effects of SAHA are reversible upon removal of the drug, the synergistic effects observed by clonogenic assays are more likely due to a potentiation of epirubicin by SAHA, than an additive effect of the two drugs.

We evaluated the potential synergy between HDAC inhibitors and other drugs and found that synergistic activity was not limited to the topo II inhibitors but was also seen with the

topo I inhibitors. In contrast, synergistic activity was not observed with other agents that do not directly interact with the DNA, irrespective of schedule.

We found that sensitivity of breast cancer cells to topo II inhibitors was related to the expression of topo II, as has been reported in the literature. Our experiments showed that the topo II depleted MDA-361 cells were resistant to epirubicin. While the nuclear concentration of epirubicin in the presence of SAHA was minimally increased, this did not result in an increase in epirubicin-induced DNA damage or apoptosis. These cells have depleted levels of topo II, but their topo I expression is unaltered and these cells were sensitized to the topo I inhibitor, topotecan. Potentiation of topo II inhibitor-induced cell death by HDAC inhibitors may, therefore, not only required chromatin decondensation, but also the expression of the targets of topo inhibitors.

In summary, we have shown that treatment of breast cancer cells with the HDAC inhibitor, SAHA, resulted in significant potentiation of epirubicin. Our findings suggest that preexposure of cells to an HDAC inhibitor leads to chromatin decondensation, rendering the DNA a better substrate for topo inhibitors. However, chromatin decondensation may not be sufficient in the absence of the target. These findings may be of clinical significance as several HDAC inhibitors are currently under clinical investigation. The results of this study may impact the optimal scheduling of the drug administration and suggest a role of determining topo IIα as a predictive marker of response.

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