Inhibition of Histone Deacetylase Increases Cytotoxicity to Anticancer Drugs Targeting DNA

Myoung Sook Kim, Melissa Blake, Jin Hyen Baek, Glenda Kohlhagen, Yves Pommier, and France Carrier

Biochemistry and Molecular Biology Department, School of Medicine, University of Maryland, Baltimore, Maryland 21201-1503 [M. S. K., M. B., F. C.]; Johns Hopkins University, Baltimore, Maryland 21231 [J. H. B.]; and National Cancer Institute, NIH, Bethesda, Maryland 20892 [G. K., Y. P.]

ABSTRACT

Several anticancer drugs target DNA or enzymes acting on the DNA. Because chromatin DNA is tightly compacted, accessibility to the drug target may reduce the efficiency of these anticancer drugs. We thus treated four human cancer cell lines and two normal epithelial cell lines with either trichostatin A (TSA) or SAHA, two histone deacetylase inhibitors, before exposing the cells to VP-16, ellipticine, camptothecin, doxorubicin, cisplatin, 5-fluorouracil, or cyclophosphamide. Pretreatment with TSA or SAHA increased the killing efficiency of VP-16, ellipticine, doxorubicin, and cisplatin. The magnitude of sensitization is cell type specific and is >10-fold for VP-16 in D54, a brain tumor cell line intrinsically resistant to topoisoamerase II inhibitors. Topoisomerase II levels and activity were not affected by this treatment, but p53, p21, and Gadd45 protein levels were markedly induced. Moreover, pretreatment with TSA also increased VP-16-induced apoptosis in a p53-dependent and -independent manner. Treating the cells in the reverse order (anticancer drug first, followed by TSA or SAHA) had no more cytotoxic effect than the drug alone. These data suggest that loosening-up the chromatin structure by histone acetylation can increase the efficiency of several anticancer drugs targeting DNA. This may be advantageous for treating tumors intrinsically resistant to these drugs.

INTRODUCTION

The packing of DNA into nucleosomal units is restrictive to most cellular processes such as transcription, replication, and repair. The Topo enzymes play important roles in these processes. However, chromatin structure prevents the Topo II enzyme from accessing the DNA and incites it to act at the linker regions (1). The nuclear Topo enzymes are responsible for regulating and maintaining DNA topology. By breaking and religating cDNA strands, they reduce torsional stress encountered during cellular metabolism (2). The Topo enzymes are thus important for proliferating cells, including cancer cells. Several classes of anticancer agents target the Topo enzymes. A number of these agents can inhibit the reversible Topo-DNA interaction and thus convert the endogenous enzyme into a cellular poison by locking the enzyme on the DNA (3, 4). Because the inhibitors function by locking the enzyme on the DNA, we hypothesize that increased accessibility of the enzyme to the chromatin DNA could result in increased drug sensitivity. Cytotoxicity to Topo inhibitors is higher during the replicative phase (S phase) of the cell cycle. This is apparently due to high Topo activity during this phase (5) and conversion of the covalent Topo DNA adducts into irreversible damage by the replication process. We propose that the looser chromatin structure encountered during replication may also contribute to increase the inhibitors’ sensitivity. Mitotic chromatin is highly condensed and transcriptionally inactive, but the active chromatin of the interphase and S phase is more decondensed and favors both transcription and replication. Moreover, newly formed chromatin after replication fork passage may remain transiently hyperacetylated and depleted in histones H2A and H2B (6), which could also facilitate accessibility of the Topo enzymes to the DNA and increase the trapping of the Topo-DNA complexes by the inhibitors.

The in vivo site selectivity of Topo cleavage is determined by several factors including chromatin structure (1, 7). Thus, the DNA availability of these sites could directly impact the cytotoxicity of the Topo inhibitors by increasing the number of enzyme molecules that can be locked on the DNA. Indications that chromatin modulation could influence Topo activity have been provided by studies on the minor groove binder distamycin (7). This antibiotic was shown to increase Topo II cleavage by a possible redistribution of the histone H1 (7, 8).

DNA accessibility can be altered by several mechanisms including multiprotein complexes that can either stabilize or destabilize nucleosome structure (reviewed in Refs. 9 and 10). Alternatively, acetylation of the core histones has been shown to weaken the histone-DNA interactions and consequently increase DNA accessibility (11, 12). In this report, we show that pretreatment of four human cancer cell lines with either TSA or SAHA before treatment with anticancer drugs that target the Topo enzymes or DNA increases the killing efficiency of all these drugs except CPT. The magnitude of sensitization is cell type specific and is >10-fold for VP-16 in D54, a brain tumor cell line intrinsically resistant to Topo II inhibitors. Topo II levels and deacetylation activity were not affected by this treatment, whereas p53, Gadd45, and p21 protein levels increased. Moreover, a TUNEL assay and a caspase 3 activation assay indicate that TSA treatment increased VP-16-mediated apoptosis in a p53-dependent and -independent manner. No synergistic effects were observed when the cells were treated in the reverse order (anticancer drugs first, followed by HDAC inhibitors). These data suggest that relaxation of the chromatin structure by histone acetylation increases the cytotoxicity of several drugs targeting the DNA. This may represent an advantage for the treatment of tumors resistant to these agents.

MATERIALS AND METHODS

Cells and the Treatments of Cells. The human glioblastoma cell line D54 (13) was provided by Drs. Eugene S. Kleinerman and Frederick F. Lang (The University of Texas M. D. Anderson Cancer Center, Houston, TX); the U118 cells, the breast cancer MCF-7 cells, and the normal breast (MCF-12F) and intestinal (FHs 74 Int) epithelial cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). The glioblastomas cells were grown in MEM containing 10% FBS (Life Technologies, Inc., Rockville, MD). The MCF-7 cells were grown in RPMI 1640 containing 10% FBS. The MCF-12F cells were grown in a 1:1 mixture of DMEM and Ham’s F-12 containing 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 0.01 mg/ml insulin, 500 ng/ml hydrocortisone, and 5% Chelex-treated horse serum. The FHs 74 Int cells were grown in Hybri-Care media (ATCC) containing 10% FBS. The human colon carcinoma RKO cells were obtained from Dr. Michael
Kastan (St. Jude Children’s Research Hospital, Memphis, TN). The RKO-E6 cells were provided by Al Fornace (National Cancer Institute, Bethesda, MD). The RKO and RKO-E6 cells were grown in RPMI 1640 containing 10% FBS in the absence of antibiotics. Clonogenic survival assays were performed in the presence of different drug concentrations. The cells were plated at 2–3 × 10^2 cells/60-mm-diameter Petri dish and exposed to either 10 ng/ml TSA (Sigma, St. Louis, MO) or 1.25 μM SAHA (Biomol, Plymouth Meeting, PA) for 4 h or left untreated before exposure to the anticancer drugs. After removing the HDAC inhibitors, the cells were exposed to increasing amounts of VP-16 (100–1000 μM), ellipticine (0.25 μM), doxorubicin (0–10 μM), cisplatin (0–10 μg/ml), CPT (0–10 μM), cyclophosphamide (0–10 mM), or 5-FU (0–10 μM) for 1 h. The anticancer drugs (all from Sigma) were then washed away, and the cells were allowed to grow for 2 weeks. The colonies were fixed with methanol-acetic acid (3:1), stained with crystal violet (0.4%), and counted. The survival fraction was determined by dividing the number of colonies formed in untreated control cells by the number of colonies formed in the treated control cells. The plating efficiency of the untreated control cells was around 50%. Each dose was done in triplicate, and the experiments were repeated at least twice.

**MTT Assays.** The plates were plated at 1 × 10^4 cells/24-well plate, and 72 h after the indicated treatments, the cells were incubated with the tetrazolium salt MTT (0.25 mg/ml in PBS) for 3 h. The media were then removed, and DMSO (1 ml of 100%) was added to solubilize the MTT-formazan product. The amount of violet crystals reflecting cellular growth and viability was determined by absorbance at 540 nm. Each sample was assayed at least three times. The results were expressed as a percentage of MTT reduction in treated samples compared with untreated sample (100%) for the drug alone or compared with TSA-treated sample (94% of untreated sample) for TSA plus anticancer-drug-treated samples.

**Western Blot Analyses.** Whole cell lysates were separated on 10–15% SDS-PAGE and transferred to nitrocellulose membrane with a semidyctse blotting apparatus (Bio-Rad, Richmond, CA). The acid-soluble histone proteins were extracted as described below. The blots were incubated with either anti-Topo II monoclonal antibody (Oncogene, Cambridge, MA) at a 1:1000 dilution, anti-acetylated H4 rabbit polyclonal antibody (Upstate Biotechnology, Lake Placid, NY) at a 1:2000 dilution, anti-p53 monoclonal antibody (Pab2412; Oncogene) at a 1:1000 dilution, anti-p21 rabbit polyclonal antibody (PharMingen, San Diego, CA) at a 1:5000 dilution, anti-Gadd45 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz Biotechnology, CA) at a 1:500 dilution, anti-PCNA monoclonal antibody (Santa Cruz Biotechnology) at a 1:1000 dilution, or anti-actin polyclonal antibody (Sigma) at a 1:5000 dilution for 2 h at room temperature. After washing, the blots were reacted with their respective secondary antibody at a 1:5000 dilution and detected with enhanced chemiluminescence reagents (Amersham) according to the supplier’s protocols. For Topo II detection, nuclear proteins were extracted and used instead of whole cell lysate.

**Extraction of Acid-Soluble Proteins.** For detection of acetylated histone H4, acid-soluble proteins were extracted with the following procedure, as recommended by Upstate Biotechnology. Briefly, the cells were pelleted down, washed with PBS, and resuspended in 5–10 volumes of cell pellets in histone lysis buffer [10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 5 mM DTT, and 1.5 mM phenylmethylsulfonyl fluoride]. Sucrose acid was then added to a final concentration of 0.2 M in polypropylene tubes on ice. After 30 min, the tubes were centrifuged at 11,000 × g for 10 min at 4°C, and the acid-soluble fraction was dialyzed against 0.1 M acetic acid twice for 1–2 h each time. The lysates were dialyzed again three more times against distilled water for 1, 3, and 16 h, respectively. Anti-acetyl histone H4 antibody was used for the detection of histone acetylation and survival assays were performed with TSA treatments.

**Apoptosis Detection.** Fragmented DNA was detected by DNA ladder formation and end-labeling of free 3′-OH. The suicide track DNA ladder isolation kit (Oncogene) was used as recommended by the manufacturer. The cells were treated as indicated in the colony formation assays, and fragmented DNA was extracted 24 h after treatments. The DNA was run on a 1.5% agarose gel and stained with ethidium bromide. Detection of the free 3′-OH was performed with the DeadEnd fluorometric TUNEL system (Promega, Madison, WI) according to the manufacturer’s protocol, 24 h after the indicated treatments. Fluorescence was detected with an inverted fluorescence microscope (Nikon TE 200, HG-100W mercury lamp).

**Caspase 3 assay (Promega)** was performed according to the manufacturer’s recommendation. The assay was performed in triplicate on 50 μg of cellular extracts. The specific caspase 3 inhibitor Ac-DEVD-CHO was used. Caspase activity was measured 12 h after treatment, and fluorescence of the cleaved substrate was read on a Wallac Victor-1420 microtiter plate fluorometer reader.

**Nuclear Extracts and Topo II Assay.** Cells were washed with ice-cold PBS and lysed by incubating them for 10 min in isotonic buffer (150 mM NaCl, 5 mM MgCl2, 2 mM KH2PO4, 1 mM EGTA, 10% glycerol, 0.1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride) containing 0.3% Triton X-100. The nuclear proteins were then extracted for 30 min in lysis buffer containing 350 mM NaCl. The extracts were centrifuged at 20,000 × g at 4°C. The protein content of the supernatant was quantified using the Bradford method (Bio-Rad). The Topo II decatenation activity was measured with a Topo II assay kit (Topogen, Columbus, OH) as recommended by the manufacturer. Kineto-plast DNA was used as a substrate, and the Topo II source was provided by the different nuclear extracts.

**DNA-Protein Cross-Links.** The DNA-protein cross-links were measured by alkaline elution as described previously (14, 15). The cells were metabolically labeled with [3H]thymidine (0.04 μCi/ml) for 36–48 h at 37°C. The cells were then chased with fresh media for at least 4 h before drug treatment. The cells were then treated with TSA and VP-16 as described in the text.

**p53 Sequencing.** Total RNA was isolated using Trizol reagents (Life Technologies, Inc.) according to the manufacturer’s protocols. Five μg of DNA-free total RNA were converted to cDNA with SuperScript II reverse transcriptase and oligo(dT)15 (Life Technologies, Inc.). Equal amounts of cDNA were subsequently amplified by PCR in a 50-μl reaction volume containing 1× PCR buffer, 200 μM deoxynucleotide triphosphates, 10 μM specific primers for human p53 (sense, 5′-CAG-AAA-ACC-TAC-GAC-AGC-3′ and antisense, 5′-TTT-CTT-CCG-GAG-ATT-CTC-3′) covering the entire p53 DNA binding domain (residues ~100 to ~300 (16)), and 1.25 units of Taq DNA polymerase (Promega). The PCR product was sequenced directly using the ABI PRISM Dye terminator cycle sequencing ready kit protocol with automated DNA Sequencer (Perkin-Elmer ABI PRISM model).

## RESULTS

**HDAC Inhibitors Increase the Efficiency of Anticancer Drugs.** To test our hypothesis that increased chromatin accessibility could increase the efficiency of drugs targeting the DNA or enzymes acting on the DNA, we first treated human glioblastoma D54 cells with TSA, a HDAC inhibitor, before treating the cells with VP-16 (etoposide, a Topo II inhibitor). The D54 cells were selected for this study based on their intrinsic resistance to Topo II inhibitors (17). The dose and time of TSA treatments were selected to obtain enough histone acetylation to confer an open chromatin structure without triggering apoptosis or genome instability. Previous reports have shown that a dose of 10 ng/ml TSA is sufficient to generate di- and tri-acetylated histones (18) without causing significant apoptosis (19). This level of acetylation correlates with increased DNase I sensitivity in chromatin (20) and thus corresponds to a more accessible, open, chromatin structure. Our data (Fig. 1A) indicate that treatment of the cells with VP-16 alone decreased survival at every dose, with a maximum effect at 25 μM VP-16 resulting in 22% survival. However, pretreatment of the cells with TSA increased VP-16 killing efficiency at every dose, with a maximum effect seen at 25 μM VP-16, where >98% of the cells were killed. This represents a 10-fold (1-log kill) increase in VP-16 killing efficiency. To determine whether histone acetylation was a requirement to increase VP-16 efficiency, we treated the cells in the reverse order. When the cells were treated with VP-16 first and then treated with TSA, no significant difference was observed compared with VP-16 treatment alone (Fig. 1A). Survival in the presence of TSA alone (10 ng/ml) was 94%.

The effect of TSA on the Topo I inhibitor CPT was also evaluated. Our data (Fig. 1B) indicate that pretreatment of D54 cells with TSA did not significantly affect CPT killing efficiency, whereas treating the cells with CPT first followed by TSA treatments slightly de-
HDAC INHIBITORS INCREASE ANTICANCER DRUG CYTOTOXICITY

Fig. 1. A, clonogenic survival after treatment with VP-16 alone, pretreatment with 10 ng/ml TSA for 4 h before VP-16 treatment, or VP-16 treatment followed by TSA in human glioblastoma D54 cells. Colonies were counted 2 weeks after treatment. Survival is expressed as the percentage of colonies obtained with untreated cells or TSA alone. B, clonogenic survival as described in A, except that cells were treated with CPT. C, clonogenic survival as described in A, except that treatments were performed in human glioblastoma U118 cells. D, clonogenic survival assessed in human colon carcinoma RKO cells as described in A.

The effect of SAHA, a HDAC inhibitor structurally related to TSA and currently under study in clinical trials, was also evaluated. A dose of 1.25 $\mu$M SAHA for 4 h was selected because under these conditions, SAHA can increase histone acetylation without affecting cell cycle progression in MCF-7 cells (22). As reported previously (22), treatment of the cells with SAHA alone had no significant effect on MCF-7 cells. Survival of 95% was consistently observed under these conditions. Like TSA, pretreatment of the cells with SAHA increased VP-16 killing efficiency. At a dose of 25 $\mu$M VP-16, survival of MCF-7 cells was reduced 4× when the cells were pretreated with SAHA (Fig. 2C). Treating the cells in the reverse order (VP-16 first, followed by SAHA) had no more effect than that seen in D54 cells. Therefore, acetylation of core histone by the HDAC inhibitors apparently has to precede the contact of the anticancer drugs with the DNA to obtain a synergistic effect.

We then asked whether the TSA effect was specific for VP-16 or could also affect other Topo II inhibitors. The data shown in Fig. 2, A and B, indicate that pretreatment with TSA also increases ellipticine and doxorubicin killing efficiency in U118 cells. The cytotoxicity of ellipticine was increased 3×, whereas doxorubicin killing efficiency was increased 7× at the highest doses of anticancer drugs used. Again, treating the cells in the reverse order (TSA first, followed by anticancer drugs) had no more effect than the anticancer drug alone. These data indicate that the synergistic effect of TSA is not drug specific and can be applied to a variety of Topo II inhibitors. Interestingly, among the three drugs tested in U118 cells, doxorubicin is the one that shows the most sensitivity to pretreatment with TSA. Doxorubicin is a DNA groove binder and an intercalating agent, whereas ellipticine is a pure intercalator, and VP-16 is not known to bind DNA (21). Close interaction of doxorubicin with DNA might thus be more responsive to a looser chromatin structure.

The effect of SAHA, a HDAC inhibitor structurally related to TSA and currently under study in clinical trials, was also evaluated. A dose of 1.25 $\mu$M SAHA for 4 h was selected because under these conditions, SAHA can increase histone acetylation without affecting cell cycle progression in MCF-7 cells (22). As reported previously (22), treatment of the cells with SAHA alone had no significant effect on MCF-7 cells. Survival of 95% was consistently observed under these conditions. Like TSA, pretreatment of the cells with SAHA increased VP-16 killing efficiency. At a dose of 25 $\mu$M VP-16, survival of MCF-7 cells was reduced 4× when the cells were pretreated with SAHA (Fig. 2C). Treating the cells in the reverse order (VP-16 first, followed by SAHA) had no more effect than that seen in D54 cells. Therefore, acetylation of core histone by the HDAC inhibitors apparently has to precede the contact of the anticancer drugs with the DNA to obtain a synergistic effect.

We then expanded our study to two other anticancer drugs currently used in cancer treatment, 5-FU and cyclophosphamide. 5-FU is an antimetabolite that can be inserted in either RNA or DNA. The state...
of chromatin compaction is thus not expected to influence its efficiency. Indeed, our data indicate (Fig. 3A) that pretreatment of MCF-7 cells with SAHA had no significant effect on 5-FU killing efficiency in these cells. The last drug that we tested is cyclophosamide. This nitrogen mustard derivative is a bifunctional alkylating agent that causes interstand DNA cross-link. Although cyclophosamide is a cross-linking agent like cisplatin (Fig. 2D), no significant effect on pretreatment with SAHA was observed in MCF-7 cells (Fig. 3B). This may be due to the fact that cyclophosphamide usually requires activation by hepatic microsomal P450 enzymes, or it may indicate that not all cross-linking agents could be affected by pretreatment with HDAC inhibitors.

To determine whether pretreatments with the HDAC inhibitors were toxic to normal cells, we repeated the assays in normal human breast (MCF-12F) and intestinal (FHs 74 Int) epithelial cells. Data obtained with cisplatinum and cyclophosphamide in MCF-12 cells are shown in Fig. 3, C and D. No significant effects on the killing efficiency of these drugs or on VP-16 and 5-FU (data not shown) were observed with pretreatments of the cells with SAHA. Similar results were obtained with the normal intestinal epithelial cells (Fig. 4), where no significant effect of pretreatment of the cells with TSA was observed with all of the drugs tested. These data indicate that pretreatments of cancer cells with HDAC inhibitors specifically increase the cytotoxicity of the anticancer drugs in cancer cells.

Cytotoxicity was also evaluated by measuring mitochondrial metabolic activity in short-term colorimetric assays (MTT) in the two glioblastomas (D54 and U118) and the MCF-7 breast cancer cell line. Our data (data not shown) indicate that TSA or SAHA alone had no cytotoxic effect. In D54 cells, TSA pretreatment of the cells before VP-16 treatments resulted in increased cytotoxicity at all VP-16 doses. Addition of TSA after VP-16 treatments did not increase the VP-16 cytotoxic effect. Comparable effects were observed in U118 cells; these cells were more resistant to VP-16 but still responded to TSA pretreatment in a manner similar to that of D54 cells. In both D54 and U118 cells, about twice as many cells were killed at every dose of VP-16 when the cells were pretreated with TSA. Cytotoxic effects were also evaluated for CPT in D54 cells (data not shown), and as shown above with clonogenic survival (Fig. 1B), no significant differences were observed between the different treatments in D54 cells. Pretreatment of MCF-7 cells with SAHA (data not shown) also increased cisplatin cytotoxicity to a greater extent than treating the cells in the reverse order. For example, about 60% of the cells survived a 5 μg/ml dose of cisplatin, whereas 3× more cells were killed when the cells were pretreated with SAHA. These data also correlate well with the clonogenic results shown in Fig. 2D.

Evaluation of Topo II Levels and Function. Cytotoxicity of Topo II inhibitors may be affected by Topo II levels and activity. Because Topo II promoter is activated by TSA (23), we wanted to determine whether the increase in sensitivity of Topo II inhibitors was due to increased Topo II levels. The levels of the nuclear Topo II enzymes were evaluated by Western blot analyses in D54 cells either immediately or 24 h after drug exposure (24 h). Our data (data not shown) indicate that the enzyme protein levels did not change in response to the different treatments either immediately or 24 h after the treatments. The levels of Topo II decatenation activity present in the cells under each treatment were also evaluated. Our data (data not shown) indicate that the kinetoplast catenated DNA substrate (cK) was fully decatenated in the untreated sample. Topo II decatenation activity was thus not affected by TSA treatment and only slightly inhibited by a 1-h treatment with VP-16. Twenty-four h after treatments, Topo II was fully active in all of the samples. These data indicate that the Topo II decatenation activity in D54 cells was not significantly affected by any of the treatments.

Effects of Treatments on Histone Acetylation and Endogenous Gene Expression. To verify that the brief TSA treatment used here was sufficient to influence histone acetylation before VP-16 treatment, we performed Western blot analysis with histone H4 acetylated specific antibody. The data presented in Fig. 5A indicate that histone H4 is acetylated after a 4-h exposure to TSA (10 ng/ml; Lane 2). TSA treatment either before or after VP-16 treatment (Lanes 3 and 5) can also increase histone H4 acetylation levels, whereas VP-16 treatment...
alone (Lane 4) had no effect. Thus, VP-16 did not prevent TSA from increasing histone H4 acetylation (Lane 3 and 5). Coomassie Blue staining of the acid-urea gel indicates that total protein loading was relatively the same in all of the lanes. As mentioned above, it is known that a dose of 10 ng/ml TSA is sufficient to generate di- and tri-acetylated histones (18). This level of histone hyperacetylation has been shown to correlate with increased DNase I sensitivity in chromatin (20). One can thus assume that under the conditions used here, acetylation of the core histone could confer an opening of the chromatin structure similar to what has been reported previously (18).

Because TSA has been shown to increase p53 levels (24) and because p53 can mediate apoptosis (25), we also evaluated the effects of TSA and VP-16 treatments on p53 levels in D54 cells. As shown in Fig. 5A, treatments with a low concentration of TSA (10 ng/ml; Lane 2) or VP-16 (10 μM, Lane 4) were sufficient to increase p53 above basal level. These data are in good agreement with previous reports using higher doses of TSA and VP-16 (17, 24). However, when the cells were pretreated with TSA and then exposed to VP-16 (Lane 3), a much higher level of p53 was observed with the same doses of drugs. Treating the cells in the reverse order [VP-16 first, followed by TSA (Lane 5)] had no more effect than each drug alone.

It is well established that the inhibition of HDAC by TSA is transient (26); thus, we aimed at determining the effect of the treatments 24 h after removal of the drugs. The data presented in Fig. 5B indicate that the levels of histone H4 acetylation were back to basal levels 24 h after exposure to the drugs. As indicated by the staining of the acid-urea gel, reduction of histone acetylation was not caused by protein loading inconsistency. The levels of p53 were still elevated after exposure to VP-16 alone (Lane 4) or TSA followed by VP-16 treatments (Lane 3) but were back to near basal levels after treatment with TSA alone (Lane 2) and VP-16 followed by TSA (Lane 5). These data suggest that brief exposure of the cells to TSA followed by VP-16 has a lasting effect on p53 levels long after the drugs have been removed and the histone acetylation levels were back to basal (hy-

---

Fig. 3. Clonogenic survival performed as described in the Fig. 1 legend with breast cancer MCF-7 cells (A and B) and normal breast epithelial MCF-12 cells (C and D) and the indicated anticancer drugs, except that SAHA (1.25 μM) was used as a HDAC inhibitor.

---

Fig. 4. Clonogenic survival performed as described in the Fig. 1 legend with normal epithelial intestinal FhS 74 Int cells and the indicated anticancer drugs.
Fig. 5. Western blots. A, the acid-soluble proteins from D54 cells were extracted immediately (No Delay) after the indicated treatments (10 μM VP-16, 1 h; 10 ng/ml TSA, 4 h). Ten μg were loaded on a 15% SDS-PAGE. Detection of acetylated histone H4 (ActH4) was performed as described in “Materials and Methods.” Even loading of protein was verified by Coomassie Blue staining of the acid-urea gel. Detection of p53 was performed on total cell extracts (50 μg) after the indicated treatments. Actin was used as a control for loading. B, detection of acetylated histone H4 and p53 was as described in A, except that the proteins were extracted 24 h after the indicated treatments. Detection of p21, Gadd45 (G45), PCNA, and actin was performed on 50 μg of total cell extracts as described in “Materials and Methods.” C, the same as B, except that the proteins were extracted from U118 cells.

The effect of VP-16 alone (Lane 4) on p53 levels also seems to last longer than the combined effect of VP-16 followed by TSA (Lane 5). We then determined the effect of the treatments on Gadd45 and p21, two p53 downstream effector genes (27). The data presented in Fig. 5B indicate that, as reported previously, p21 protein levels are increased by TSA (24) and VP-16 (28). Pretreatment of the cells with TSA before VP-16 exposure (Lane 3) increased p21 levels more than any of the treatments separately (Lanes 2 and 4), whereas the p21 levels were at basal levels 24 h after treating the cells in the opposite order (Lane 5). Interestingly, when the cells were analyzed immediately after treatments, the p21 levels of the sample treated with VP-16 first followed by TSA were comparable with levels seen after treatment with each drug separately (data not shown). Although induction of p21 by HDAC inhibitors is apparently p53 independent (29), the effects and order of the drugs treatments on p21 protein levels correlate very well with p53 expression here. The levels of Gadd45 also correlated well with p21 and p53 expression. As with p53 and p21, levels of Gadd45 were highest when the cells were treated with TSA first, followed by VP-16 (Lane 3). No significant increase in Gadd45 protein levels was observed when the cells were treated for 1 h with VP-16 alone (Lane 4). This is different than what has been reported by another group (30), where Gadd45 levels were increased after exposure to VP-16. However, in that study, the cells were treated with VP-16 for 3 h. Addition of TSA after VP-16 did not stimulate p53, p21, and Gadd45 protein levels (Lanes 5). The levels of PCNA and actin (Fig. 5B), as well as staining for total proteins, were measured as internal controls and were not significantly affected by any of the treatments. These data thus indicate that TSA followed by VP-16 (Lanes 3) is the most effective treatment to induce p53, p21, and Gadd45 protein levels. We also measured p53, p21, and Gadd45 protein levels in U118 cells treated similarly (Fig. 5C). Essentially the same results were obtained, even though the levels of p53 and p21 induction were not as high as those seen in D54 cells. Taken together, these data suggest that acetylation of the core histones has to occur before VP-16 can lock Topo II on the DNA to enhance the VP-16-induced expression of p53, p21, and Gadd45.

Pretreatment with TSA Does Not Increase the Overall Number of DNA-Protein Cross-Links. Because a change in cellular sensitivity to Topo inhibitors could be reflected on the levels of covalent linkages formed between the enzymes and the DNA (3), we measured the levels of proteins cross-links formed with VP-16 and CPT in U118 cells pretreated with TSA (Fig. 6). Our data indicate that the levels of DNA-protein cross-links formed with each inhibitor increased in a dose-dependent manner but that the levels of DNA-protein cross-links did not change consistently when the cells were pretreated for 4 h with TSA (Fig. 6). The difference between the survival data (Fig. 1) and the cross-linking data may be due to the limitation of the alkaline elution technique (14), which can measure DNA-protein cross-links in the overall genome but will not detect selective changes.

TSA Increases VP-16-Mediated Apoptosis. D54 cells are resistant to VP-16 induced apoptosis (17). Because cytotoxicity to Topo II inhibitors is often correlated with apoptosis (31) and because our data (Fig. 1) indicate that pretreatment of D54 cells with TSA increases VP-16 cytotoxicity, we wanted to determine whether the increased cytotoxicity observed in Fig. 1 affected apoptosis. We first measured the levels of internucleosomal DNA cleavage (DNA ladder) in each of the treatments used. Our data (data not shown) indicate that none of the treatments used, including pulse treatment with VP-16 alone and...
brief treatment of the cells with TSA alone or before and after VP-16 treatment, had an effect on internucleosomal DNA cleavage. However, internucleosomal cleavage occurs rather late in apoptosis stages and cannot be detected when <10–20% of the cells are apoptosing. We thus performed a more sensitive assay (DeadEnd; fluorescence TUNEL system) that allows detection of DNA strand breaks by labeling free 3'-OH termini. The data presented in Fig. 7 indicate that brief TSA treatment with doses as low as 10 ng/ml (Fig. 7f) can increase the levels of DNA strand breaks above the basal level in D54 cells. The 1-h pulse treatment with VP-16 alone (Fig. 7f) did not cause sufficient DNA strand breaks to be detectable by the TUNEL assay. Pretreatment of the cells with TSA followed by VP-16 1-h pulse increased the levels of DNA strand breaks severalfold above background (Fig. 7h). Treating the cells in the reverse order [VP-16 first, followed by TSA (Fig. 7f)] produced similar amounts of DNA strand breaks as treatment with TSA alone (Fig. 7f). These data correlate well with the cytotoxicity data presented in Fig. 1 and suggest that acetylation of the core histone before VP-16 interaction with DNA and the Topo II enzyme can increase VP-16 cytotoxicity.

Role of p53 in Mediating TSA-Induced Sensitization to Anticancer Drugs. Depending on the cell lines, functional p53 can either increase or reduce the sensitivity to anticancer drugs (32). To determine whether p53 plays a role in the TSA-induced sensitization to anticancer agents, we repeated the survival and TUNEL assays in addition to performing a caspase 3 assay in RKO-E6 cells. These cells are identical to the parental RKO cells, except that they express the viral E6 protein that degrades p53 (33). We first verified that the levels of p53 protein in the RKO-E6 cells were reduced. The data shown in Fig. 8a indicate that p53 levels are readily detectable in the RKO parental cell line (Lanes 1, 3, and 5) but are below detection levels in the RKO-E6 cellular extracts (Lanes 2, 4, and 6). However, the survival curves obtained with both cell lines pretreated with TSA before VP-16 are virtually identical (Fig. 8b). No differences between the two cell lines were noticed when the cells were treated with either VP-16 alone (data not shown) or VP-16 followed by TSA (Fig. 8c). Nonetheless, because clonogenic survival does not distinguish between the different cell death mechanisms and because functional p53 has been associated with apoptosis mediated by Topo II poisons (34), we also measured the role of p53 in the TSA sensitization to VP-16-mediated apoptosis. Both RKO and RKO-E6 cells were treated and analyzed by TUNEL assays as described above for the D54 cells. Like D54 cells, the RKO cells show increased VP-16-induced apoptosis when pretreated with TSA (data not shown). On the other hand, VP-16-induced apoptosis was not increased to the same extent by TSA pretreatment in RKO-E6 cells (data not shown). These data suggest that both p53-dependent and p53-independent apoptosis are responsible for TSA sensitization to VP-16 cytotoxicity.

As a more direct measure of apoptosis, we evaluated the activation of caspase 3 after each treatment in RKO and RKO-E6 (Fig. 9). The data shown in Fig. 9a indicate that activation of caspase 3 is markedly increased when the RKO cells are pretreated with TSA. Pretreatment with TSA also increased caspase 3 activity in RKO-E6 cells, but to a lesser extent than that seen in the wild-type cell line (Fig. 9b). These data indicate that the increased cytotoxicity observed with pretreatment of the cells with HDAC inhibitors is mediated by increased activation of caspase 3; consequently, apoptosis, part of this effect, is p53 dependent.

DISCUSSION

It has been reported recently (35) that pretreatment of drug-resistant cells with TSA increases Topo II inhibitor cytotoxicity. This effect was attributed to the interaction of Topo II with the HDAC. If this was the only contributing factor, the order of drug addition to the cultured cells should not matter. The interaction of Topo II with HDAC should be the same, and the effect they mediate on cytotoxicity should be similar. Our data suggest otherwise. The sensitization to VP-16 cytotoxicity was observed only when TSA was added before VP-16 (Fig. 1). Our data indicate that the TSA treatment used here was sufficient to acetylate histone H4 (Fig. 5a). Previous reports have shown that these conditions are sufficient to generate di- and tri-acetylated histones (18). This level of histone hyperacetylation is sufficient to neutralize the basic charges on the histone tails and confer an open chromatin structure that correlates with increased DNase I sensitivity (20). It is thus likely that the permissive, more open state of the chromatin generated by the HDAC inhibitors increased the efficiency of the anticancer drugs targeting DNA or enzymes acting on DNA. The possibility that the HDAC inhibitors act through the acetylation of other proteins including p53 cannot be ruled out. However, this possibility is not supported by the fact that addition of the HDAC inhibitors after the anticancer agents had no synergistic effect on cytotoxicity. Our data have shown that TSA is still active and can increase histone H4 acetylation even when added after VP-16 (Fig. 5). Although acetylation of proteins occurs under these conditions, no effect on the cytotoxicity of anticancer drugs was observed (Figs. 1 and 2).

Increased accessibility of the Topo inhibitors to the chromatin DNA could also result in an increased number of ternary complexes covalently trapped by Topo poisons (28, 36). Nonetheless, using alkaline elution, we could not measure any differences between the cells pretreated with TSA and the cells treated with the Topo inhibitors only (Fig. 6). However, it is possible that the number of cross-links increased only at specific sites in the chromatin and not throughout the genome. This possibility is supported by the fact that TSA acts selectively on genes, affecting the expression of only approximately
2% of expressed genes (37). The alkaline elution technique measures the DNA-protein cross-links throughout the genome and might not be sensitive enough to measure subtle differences. The induction of p53 by pretreatment of the cells with TSA (Fig. 5) might be an indication of increased Topo cleavage because a single DNA strand break is sufficient to induce p53 (38, 39). The antibiotic distamycin has been shown to selectively stimulate Topo II cleavage in vivo at a small number of consecutive nucleosome linker sites (7, 8). This effect is mediated by a local redistribution of histone H1 at these sites. Whether TSA can selectively stimulate Topo II cleavage at a defined number of sites is not known, but this remains a plausible possibility.

Under the conditions used in this study, pretreatment of cells with HDAC inhibitors seems to affect only Topo II and not Topo I inhibitors cytotoxicity (Fig. 1). This may be due to the different mechanism of action of the two enzymes and the different effects that nucleosome positioning inflicts on the two enzymes. Topo II cleavage sites are strongly suppressed in SV40 chromatin (1), whereas Topo I cleavage specificity and efficiency are the same on protein-free DNA and in vitro-assembled SV40 chromatin (40). The fact that Topo II but not Topo I associates with HDAC (41) may also indicate the importance of chromatin conformation for enzyme activity. Topo I may not need to associate itself with chromatin remodeling factor because it can sense chromatin distortion and adapt its cleavage specificity accordingly (42, 43). Moreover, chromatin structure, not sequence specificity, is apparently the primary determinant of Topo II site selection in vivo (44). Accessibility of the sites could thus have a direct impact on the cytotoxicity of inhibitors. Topo II has also been identified as a component of the chromatin remodeling complex CHRAC (45). Whereas Topo II does not seem to be involved in CHRAC activity, it has been suggested that CHRAC could help Topo II interaction with chromatin and thus enhance its function (45).

Fig. 7. Detection of free 3'-OH. The D54 cells were treated as indicated. Twenty-four h later, the cells were reacted with terminal deoxynucleotidyl transferase (TdT) and fluorescein-labeled and unlabeled deoxynucleotides. The cell populations are shown in phase-contrast (a, c, e, g, i, and k) and with a fluorescein filter (b, d, f, h, j, and l).

Fig. 8. A, Western blot analysis of RKO (−) and RKO-E6 (+) protein extracts. The blots were reacted with either p53 or actin antibody as described in “Materials and Methods.” B, clonogenic survival assessed as described in the Fig. 1 legend in RKO and RKO-E6 cells. Cells were pretreated with TSA (10 ng/ml) for 4 h before addition of VP-16. C, clonogenic survival was assessed as described in B, except that the cells were treated with VP-16 before addition of TSA.
implication that CHRAC facilitates Topo II accessibility to chromatin DNA is reminiscent of our hypothesis regarding the effect of HDAC inhibitors on Topo II accessibility.

Intrinsic resistance to Topo II inhibitors often correlates with decreased drug uptake or altered Topo II protein (46). In D54 cells, Topo II is active (this paper and Ref. 17), and drug uptake is comparable with other cell lines that are more sensitive to Topo II inhibitors (17). Other mechanisms are thus likely responsible for the resistance to Topo II inhibitors in these cells. Our data (Fig. 1C) indicate that the glioblastoma U118 cells are even more resistant to VP-16 than the D54 cells. However, pretreatment of both cells with TSA increased VP-16 killing efficiency 7–10× (Fig. 1, A and C). These data suggest that HDAC inhibitors can be used to enhance the efficiency of Topo II inhibitors in tumors that are intrinsically resistant to these drugs but have a functional Topo II enzyme.

In some cell lines, functional p53 correlates with drug cytotoxicity (47, 48). However, a role for p53 in cellular sensitivity to chemotherapeutic drugs remains controversial (34). The main source of discrepancies seems to be the techniques used to measure cell death. Cells that die by mechanisms other than apoptosis will be accounted for in a clonogenic survival assay but overlooked with apoptosis-specific assays. We used three cell lines that have a wild-type p53 genotype [D54 (49), MCF-7 (50), and RKO (51)] and one that has a mutant genotype but can still induce p53 by DNA damage [U118 (52, 53)].

We also used one wild-type cell line that had been stably transfected with the oncoprotein E6 to inactivate p53 (RKO-E6, Ref. 33). Our clonogenic survival data (Figs. 1–4 and 8) indicate that pretreatment of all of the cancer cell lines with HDAC inhibitors increased the cytotoxicity of the several anticancer drugs, regardless of p53 status in these cells. However, when the capacity to apoptose was measured, at least part of it was shown to be p53 dependent (TUNEL assay and Fig. 9). Interestingly, a recent study (41) reported that TSA inhibited VP-16-mediated apoptosis. This study used three cell lines that had an abnormal p53 genotype. Myeloid leukemia HL-60 cells and human large cell lung carcinoma H1299 cells have a null p53 genotype (51, 54), whereas the HeLa cells are infected with HPV-18, which contains an E6 protein inhibiting normal p53 function (51, 55). Cell type specificity may thus add to the complex contribution of a functional p53 in the induction of apoptosis. A previous study has reported that TSA could protect murine embryo fibroblast from p53-induced apoptosis (56). In addition to using a murine cell line, this study also used 10× more TSA for 6× longer exposure than what we used here. The different procedures may account for the different results.

Our data and the studies mentioned above suggest that at least two cell death mechanisms are mediating the sensitization of HDAC inhibitors to anticancer drugs: (a) one involves apoptosis and can be both p53 dependent and independent; and (b) one probably involves mechanisms other than apoptosis such as necrosis. These two mechanisms are apparently not mutually exclusive or synergistic because no significant differences were observed between RKO and RKO-E6 clonogenic survival (Fig. 8). The sensitization to anticancer drugs by the HDAC inhibitors could thus be applied to the treatment of a large variety of tumors, regardless of their p53 status.

In summary, our data indicate that brief treatment with HDAC inhibitors before exposure to anticancer drugs can increase drug cytotoxicity, even in cells that are intrinsically resistant to these drugs. A wild-type p53 genotype can contribute to cytotoxic effect by increasing apoptosis, but this effect is not limiting. The use of small doses of HDAC inhibitors for a short period of time before anticancer drug treatments may prove advantageous in the clinic and reduce cytotoxicity.

REFERENCES


Downloaded from cancerres.aac jintournals.org on April 13, 2017. © 2003 American Association for Cancer Research.


Inhibition of Histone Deacetylase Increases Cytotoxicity to Anticancer Drugs Targeting DNA

Myoung Sook Kim, Mellissa Blake, Jin Hyen Baek, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/63/21/7291

Cited articles
This article cites 53 articles, 24 of which you can access for free at:
http://cancerres.aacrjournals.org/content/63/21/7291.full.html#ref-list-1

Citing articles
This article has been cited by 65 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/63/21/7291.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.