The Histone Deacetylase Inhibitor Sodium Butyrate Induces DNA Topoisomerase IIα Expression and Confers Hypersensitivity to Etoposide in Human Leukemic Cell Lines

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Abstract

The differentiating agent and histone deacetylase inhibitor, sodium butyrate (NaB), was shown previously to cause a transient, 3–17-fold induction of human DNA topoisomerase IIα (topo IIα) gene promoter activity and a 2-fold increase in topo IIα protein early in monocytic differentiation of HL-60 cells. This observation has now been extended to other short chain fatty acids and aromatic butyrate analogues, and evidence is presented that human topo IIα promoter induction correlates closely with histone H4 acetylation status. Because increased topo IIα expression is associated with enhanced efficacy of topo-II-poisoning antitumor drugs such as etoposide, the hypothesis tested in this report was whether NaB pretreatment could sensitize HL-60 myeloid leukemia and K562 erythroleukemia cells to etoposide-triggered DNA damage and cell death. A 24–72 h NaB treatment (0.4–0.5 mM) induced topo IIα 2–2.5-fold in both HL-60 and K562 cells and caused a dose-dependent enhancement of etoposide-stimulated, protein-linked DNA complexes in both cell lines. At concentrations with minimal effects on cell cycle kinetics (0.4 mM in HL-60; 0.5 mM in K562), NaB pretreatment also modestly enhanced etoposide-triggered apoptosis in HL-60 cells, as determined morphologically after acridine orange/ethidium bromide staining, and substantially increased K562 growth inhibition and poly(ADP-ribose)polymerase cleavage after etoposide exposure. Therefore, a temporal window may exist whereby a differentiating agent may sensitize experimental leukemias to a cytotoxic antitumor agent. These results indicate that histone deacetylase inhibitors should be investigated for etoposide sensitization of other butyrate-responsive hematopoietic and nonhematopoietic tumor lines in vitro and in vivo.

Introduction

The transcriptional and posttranscriptional regulation of DNA topo6 IIα, a nuclear enzyme essential for completion of mitosis (1–3), has been a primary focus of our research groups (4–8). Drugs such as the epipodophyllotoxins (etoposide and teniposide), anthracyclines, ellipticines, anthracediones, and aminocycridine derivatives exert their cytotoxic activity, at least in part, by trapping topo II in a covalent complex with DNA (9). These “cleavable complexes” (10) can act as physical barriers to DNA replication (11–13), cause mitotic catastrophes (14), induce recombination events (15, 16), and/or trigger a cytotoxicity cascade culminating in apoptosis (17–19). Because the class of antitumor drugs converts this essential enzyme into a lethal instrument, it follows that tumor cell populations possessing high levels of topo IIα are more effectively killed by these agents.

Two genes exist for mammalian type II DNA topoisomerasies, termed α and β, likely attributable to evolutionary gene duplication (20, 21). Topo IIα is often the more predominant cellular form (22, 23). Cleaveble complexes formed with topo IIα correlate more closely with teniposide cytotoxicity in leukemia cells than do those formed with topo IIβ (24), but in other systems topo IIβ may also influence drug sensitivity (25, 26). The α form is expressed in a proliferation-dependent manner, whereas the β form is expressed independently of growth status (27–29). Therefore, rapidly growing cells that

9 The abbreviations used are: topo, topoisomerase; NaB, sodium butyrate; HDAC, histone deacytlate; IC50, drug concentration that inhibits growth by 50%; PARP, poly(ADP-ribose)polymerase; ATCC, American Type Culture Collection; CMV, cytomegalovirus; β-gal, β-galactosidase; SCFA, short-chain fatty acid; TSA, trichostatin A.
contain high topo IIα levels are effectively killed by the topo II-directed drugs, whereas differentiated or otherwise growth-arrested cells usually possess diminished topo IIα levels and are intrinsically resistant to these agents (9, 30, 31). Similarly, acquired resistance to a topo II-directed agent is often attributable to suppressed topo IIα and/or topo IIβ expression (8, 32, 33).

Several transcription factors have been linked to the high, proliferation-dependent expression of topo IIα including c-Myb and B-Myb (5), NF-M (4), NF-Y (34–37), and YB-1 (38). The Sp3 transcription factor is also known to activate the human topo IIα gene (39). Conversely, the p53 tumor suppressor protein represses the human topo IIα gene through the basal transcriptional machinery (40) or via an inverted CCAAT box at position –68 (41). The activated Ras pathway also stimulates both topo IIα activity and expression. topo IIα activity is enhanced via protein interactions with extracellular signal-regulated kinase 2 (42), and topo IIα trans-activation can be driven through a Ets-like enhancer element at position –480 of the topo IIα promoter (43). Finally, hyperthermia also induces topo IIα expression in vitro (44) as a result of increased topo IIα mRNA stability (45).

In previous studies, the serendipitous observation was made that topo IIα transcription and gene expression was transiently induced by the monocytic differentiating agent, NaB (6). NaB has long been recognized as an inhibitor of HDAC activity (46), an effect that is, in turn, associated with selective gene activation or repression (47–49). It was hypothesized that as a result of increased topo IIα expression, NaB-pretreated leukemia cells would exhibit increased sensitivity to etoposide because of increased topo II-mediated DNA damage.

In the present study, we show that several SCFAs and aromatic butyrate analogues also stimulate the activity of a synthetic topo IIα promoter-reporter construct in direct relation to the magnitude of histone deacetylase inhibition. NaB, the most efficacious stimulator of topo IIα promoter activity and histone H4 acetylation, also induces the endogenous expression of pharmacologically competent topo IIα protein in both HL-60 and K562 cells. Consequently, NaB pretreatment sensitizes human leukemia cells to etoposide cytotoxicity measured by increased fraction of morphologically apoptotic cells (HL-60), enhanced growth inhibition (K562), or stimulation of PARP proteolytic cleavage. Our results are discussed in light of other recent reports of a physical relationship between topo IIα and HDACs and indicate that other butyrate-responsive hematopoietic and nonhematopoietic cell lines should be investigated for synergism between HDAC inhibitors and classical cytotoxic agents.

Materials and Methods

Chemicals and Enzymes. Unless noted otherwise, all cell culture reagents and molecular biology grade enzymes were obtained from Life Technologies, Inc. (Gaithersburg, MD). All SCFAs were obtained from Sigma Chemical Co./Aldrich Chemical (St. Louis, MO) as sodium salts (acetate, propionate, butyrate, and caproate) or free acids (valeric and heptanoic acids), the latter of which were solubilized in equimolar NaOH. Solutions (in 0.9% saline) of sodium phenylacetate and sodium phenylbutyrate manufactured by Elan Pharmaceuticals (San Francisco, CA) were generous gifts of Dr. Andrew Kraft (Division of Medical Oncology, University of Colorado School of Medicine). Buffers and all other chemicals were obtained from United States Biochemical/American Pharmacia Biotech (Arlington Heights, IL). Chemicals were of the highest purity listed by the supplier, and molecular biology-tested reagents were obtained wherever possible.

Mammalian Cell Culture. HL-60 human promyelocytic cells (ATCC CCL 240) or K562 human erythroleukemia cells (ATCC CCL 243) were obtained from the ATCC (Manassas, VA). Cells were maintained as suspension cultures in either RPMI 1640 (HL-60) or Iscove’s modified Minimal Essential Medium (K562; Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 50 units/ml penicillin G, and 50 μg/ml streptomycin sulfate. All cells were maintained at 37°C in a humidified atmosphere containing 5% CO2. Cells were carried as exponentially growing cultures by propagation at 5 × 10^5 cells/ml every 2–3 days.

Cellular Histone Acetylation Assay. Nuclei were isolated, and histone fractions were prepared from cell nuclei as described (50) with the following modifications. Pelleted nuclei were resuspended in 1.35 ml of sterile, ice-cold water containing 1 mM phenylmethylsulfonyl fluoride and 0.15 ml of 4 n H2SO4 added dropwise with swirling and histones extracted overnight. After centrifugation at 12,000 × g for 10 min, histone supernatants were transferred to 15 ml of Falcon polypropylene tubes and filled with cold acetone/HCl (99:1 acetone:5 n HCl) and incubated at −20°C for 72 h. During the final pelleting of the histone isolates, all samples were initially centrifuged for 10 min at 2,000 × g in a swinging-bucket rotor. The supernatants were aspirated to ~1 ml, mixed by pipetting, then transferred to a 1.5 ml of Eppendorf tube for centrifugation at 12,000 × g for 10 min at 4°C. Supernatants were carefully aspirated, and pellets were lyophilized and then resuspended in loading buffer (8 M urea, 10% glycerol, 0.9 n acetic acid, 5% 2-mercaptoethanol, and 0.25% methyl green) for Triton-acid-urea gel electrophoresis. Triton-acid-urea gels containing 12% polyacrylamide and run for another hour at 115 V. Solubilized histones were boiled for 5 min and then loaded and subjected to electrophoresis at 115 V for 15–18 h. After Coomasie Blue staining, gels were scanned, and acetylated bands were quantified on a Bio-Rad Fluor-S Multimager (f/11, white light scan, clear filter, 3 s). The intensity of un-, bi-, and triacetylated histone H4 bands (denoted 1, 2, and 3, respectively) were combined, and data were expressed as a ratio relative to the unacetylated form (denoted 0).

Topo II Assays. Western immunoblotting for steady-state topo IIα protein levels from HL-60 nuclear extracts was performed exactly as described by Fraser et al. (6) using a polyclonal antiserum generated in the Yalowich laboratory to a COOH-terminal recombinant topo IIα peptide (7).
Transcriptional activation of a topo IIα promoter-luciferase reporter construct was assessed following cellular electroporation as described previously (6) using the construct, −562TOP2LUC. The promoter region of this plasmid corresponds to positions −562 to +92 of the human topo IIα 5′-flanking region (51), which had been cloned upstream of the firefly luciferase cDNA in pA3LUC. Various concentrations of NaB or other fatty acids (dissolved in sterile water, or 0.9% saline for the aromatic analogues) were incubated in triplicate with transfected cells continuously for 24 h prior to harvest and quantitation of reporter gene activities. topo IIα promoter activity was normalized to the activity derived from an internal control plasmid, pCMV-βgal, which encodes Escherichia coli β-gal under control of the CMV immediate/early promoter, as described previously (5, 6).

Etodiposide-stabilized topo II-DNA cleavable complexes were quantified using a modification (32) of a K-SDS precipitation assay. Briefly, exponentially growing HL-60 or K562 cells (5 × 10⁵/ml) were incubated for 24 h with [¹⁴C]leucine (0.2 μCi/ml; 325 mCi/μmol) and [methyl-³H]thymidine (0.6 μCi/ml; 6.7 Ci/mmol). In the case of HL-60 cells, the labeling was accompanied by a 24-h exposure to either 0 or 0.4 mM NaB; for K562 cells, the labeling was preceded by a 48-h exposure to 0 or 0.5 mM NaB and continued during the labeling period for a total of a 72 h butyrate exposure. During the final 30 min, cells received 0–100 μM etoposide (Sigma Chemical Co.; dissolved in DMSO) or an equivalent amount of vehicle, in triplicate. Vehicle was ultimately expressed as a percentage of inhibition of control cell growth. The 50% growth inhibitory concentration (IC₅₀) was calculated from replicate concentration-response curves generated from three separate experiments.

Cleavage of PARP as a measure of apoptotic caspase activity (53) was also assessed after etoposide exposure in K562 cells pretreated with NaB (0.5 mM for 72 h) by immunoblotting for the M₉116,000 parent band and the M₉85,000–89,000 proteolytic cleavage product. Following SDS-PAGE with 7.5% acrylamide and electrophertransfer to Immobilon-P membranes, blots were probed with a polyclonal antisemur obtained from Santa Cruz Biotechnology Biochemical (Santa Cruz Biotechnology, CA) at a 1:1000 dilution with a1:5000 dilution of goat antirabbit-horseradish peroxidase conjugate (Pierce, Rockford, IL), and bands were detected by fluorescent radiography using an enhanced chemiluminescence substrate (NEL).

**Statistical Analysis.** Where indicated, differences from control values were assessed by one-way ANOVA using Dunnett’s multiple comparison post-hoc test with GraphPad Prism software. Significant differences were indicated where P < 0.05.

**Results**

Several SCFA Induce Both the Activity of the DNA topo IIα Promoter and Acetylation of Histone H4 in HL-60 Cells. It has been recognized for >20 years that SCFAs other than NaB are capable of enhancing the acetylation state of histones, especially histone H4, by inhibiting cellular histone deacetylase activity (46, 54). Having initially demonstrated that NaB was an efficacious inducer of the human topo IIα promoter (6), we sought to investigate whether this was a property shared by other SCFAs and, if so, whether the magnitude of induction correlated with HDAC inhibitory activity. HL-60 cells were transfected with −562TOP2LUC and treated for 24 h with 1 mM concentrations of each of the
SCFAs with carbon chain lengths from two to seven and the aromatic butyrate analogues, phenylacetate and phenylbutyrate (as sodium salts), followed by quantitation of luciferase reporter enzyme activity. Parallel cultures, treated identically, were subjected to analysis of histone acetylation status by electrophoresis on Triton-acid-urea gels.

SCFAs as short as acetate (C2) produced a significant increase in topo IIα promoter activity, which rose substantially as chain length increased, peaking at 9.6-fold with butyrate (C4), then decreasing to 4.3-fold with caproate (C6), and returning to control levels with the seven-carbon fatty acid, heptanoate (Fig. 1). Of the aromatic butyrate analogues, only phenylbutyrate substantially activated this promoter construct in HL-60 cells, although phenylacetate could induce the promoter \( \geq 2 \)-fold at concentrations \( \geq 4 \) mM (data not shown). The biphasic response seen with the SCFAs is highly reminiscent of the relationship between carbon chain length and histone acetylation observed previously in HeLa cells (54). We therefore investigated whether there was a direct correlation between the magnitude of topo IIα promoter activation produced by the SCFAs and the increase in histone H4 acetylation status.

When similarly treated HL-60 cells were subjected to histone acetylation analysis (Fig. 2A), it was clear that the most efficacious topo IIα promoter inducers were also the most effective inhibitors of histone H4 deacetylation. This relationship is demonstrated graphically in Fig. 2B. However, this concordance with topo IIα promoter activation appeared to plateau at the very high levels of histone acetylation caused by butyrate. This observation suggests an upper limit to this relationship, such that above an acetylated:unacetylated histone H4 ratio of 3, little further induction of the topo IIα promoter can be achieved.

Our previous work demonstrated that 0.4 mM NaB treatment of HL-60 cells for 24 h induced topo IIα promoter-driven reporter enzyme activity 3–17-fold but caused only a \(-2\)-fold increase in steady-state topo IIα protein levels (6). A similar magnitude of NaB induction of topo IIα protein levels (2.1 \( \pm \) 0.3-fold; mean \( \pm \) SE from three separate experiments) is shown in Fig. 3 by the band at \( M_0 \) 170,000. Using 24 h pretreatment as the optimal time for observing topo IIα induction, we investigated whether greater NaB concentrations (1–8 mM) would further induce enzyme expression. Although these higher NaB concentrations caused a 15–40-

Fig. 1. Effect of SCFAs and butyrate analogues on topo IIα promoter activity in HL-60 cells. A, the topo IIα promoter-luciferase reporter plasmid, p5621TOP2LUC (20 \( \mu \)g), and an internal control plasmid, pCMV-\( \beta \)-gal, were cotransfected into HL-60 cells by electroporation, and cells were exposed to the sodium salts of the indicated fatty acids [acetate (C2), propionate (C3), butyrate (C4), valerate (C5), caproate (C6), heptanoate (C7), phenylacetate, or phenylbutyrate] at a concentration of 1 mM for 24 h. Cells were then processed for measurement of luciferase activity, and expression was normalized to that of \( \beta \)-galactosidase activity produced from the internal control construct. Data are expressed as relative light units divided by milliunits of \( \beta \)-gal activity; bars, SE.

Fig. 2. Correlation of increased histone H4 acetylation with topo IIα promoter activation. A, each group of HL-60 cells was treated with each of the fatty acids (1 mM) for 24 h as in Fig. 1. Cells were then processed for isolation of nuclear histones as described in “Materials and Methods,” subjected to electrophoresis on a Triton-acid-urea polyacrylamide gel, and stained with Coomassie Blue. Acetylation of histone H4 was quantified by scanning the intensities of the unacetylated band and the total acetylated bands. The intensity of uni-, bi-, and triacylated histone H4 bands (denoted 1, 2, and 3, respectively) were combined, and data were expressed as a ratio relative to the unacylated form (0). Differences in loading and/or histone recovery was therefore normalized by expressing acetylation as the ratio of total acetylated H4 unacylated H4. B, graphic representation of the relationship between histone H4 acetylation and topo IIα promoter activation. The data from Fig. 1 (Y axis) and A in this figure (X axis) are plotted with each point denoted by the associated fatty acid treatment. PA, sodium phenylacetate; PB, sodium phenylbutyrate.
increase in topo II levels. Logarithmically growing leukemia cells were exposed to NaB (0.4 mM, 24 h for HL-60; 0.5 mM, 72 h for K562) and then lysed directly in SDS loading buffer. Proteins were resolved by electrophoresis on SDS-7.5% acrylamide gels, transferred to Immobilon-P membranes, and then immunoblotted for human topo II using an antiserum raised to the COOH terminus of the enzyme. Immunoreactivity was visualized using a goat antirabbit IgG-horseradish peroxidase secondary antibody and chemiluminescent substrate. topo II bands were quantified by scanning on a Bio-Rad Fluor-S imaging system.

fold induction of reporter enzyme activity derived from the promoter construct, endogenous topo II protein levels still increased by only 2.5–2.7-fold at 1 mM NaB and fell to under 2-fold control levels at higher NaB concentrations (data not shown). These results are not entirely surprising in that topo II levels rarely vary by more than 2–3-fold in proliferating cells, and forced overexpression of the enzyme is known to cause feedback inhibition of its own transcription (55).

We next sought to learn whether NaB could induce topo II protein levels in another commonly used experimental leukemia cell line, K562. Similar to HL-60 cells, K562 cells will differentiate in response to NaB but exhibit more delayed kinetics in both differentiation as well as in response to cytotoxic agents (18). In K562 cells treated with NaB for 72 h, topo II expression was induced 2.5 ± 0.2-fold (mean ± SE from five separate experiments; Fig. 3).

Increased topo II Expression in HL-60 and K562 Cells Correlates with Increased Etoposide-stabilized, Protein-linked DNA. To assess whether the topo II protein induced by NaB in HL-60 and K562 cells was pharmacologically functional, a classic protein-linked DNA assay was used in the presence of etoposide as an indicator of the extent of cleavable complex formation in intact cells (Fig. 4). Consistent with the NaB-mediated increase in topo II expression in HL-60 and K562 cells (Fig. 3), NaB-pretreated HL-60 and K562 cells both displayed a greater magnitude of protein-linked DNA as a function of etoposide concentration, as compared with control cells not exposed previously to NaB (Fig. 4). Therefore, the increased levels of topo II detected in Fig. 3 represented a pharmacologically functional pool of enzyme in both leukemic cell lines. However, it should be noted that one limitation of this assay is the inability to determine the topo II isoform increasingly trapped in complexes from NaB-pretreated cells. Although these data are consistent with an increase in topo II, conclusive demonstration of the isoform(s) involved will require immunological confirmation.

Fig. 3. NaB treatment of HL-60 and K562 cells increases topo II protein levels. Logarithmically growing leukemia cells were exposed to NaB (0.4 mM, 24 h for HL-60; 0.5 mM, 72 h for K562) and then lysed directly in SDS loading buffer. Proteins were resolved by electrophoresis on SDS-7.5% acrylamide gels, transferred to Immobilon-P membranes, and then immunoblotted for human topo II using an antiserum raised to the COOH terminus of the enzyme. Immunoreactivity was visualized using a goat antirabbit IgG-horseradish peroxidase secondary antibody and chemiluminescent substrate. topo II bands were quantified by scanning on a Bio-Rad Fluor-S imaging system.

Fig. 4. NaB effects on etoposide-mediated, protein-linked DNA in HL-60 and K562 cells. A standard K-SDS precipitation assay was used to measure etoposide-stabilized, cleavable complex formation. HL-60 cells (A; n = 3/group) and K562 cells (B; n = 4 with separate experiments performed on separate days) were either left untreated (no NaB) or exposed to 0.4 or 0.5 mM NaB, respectively, as in Fig. 3, and labeled for the final 24 h with [3H]thymidine (0.6 μCi/ml) and [3H]thymidine (0.6 μCi/ml). Cells were then treated with the indicated concentrations of etoposide (or vehicle) for 30 min and lysed, and protein-DNA complexes were precipitated as described in “Materials and Methods.” Protein-linked DNA was quantitated by liquid scintillation counting to generate protein-linked DNA as a ratio of [3H]cpm/125I cpm. The data are expressed as the mean fold-increase in this ratio relative to each DMSO vehicle control; bars, SE. □, no NaB; △, 0.4 mM NaB (A) and 0.5 mM NaB (B).

NaB Confers Hypersensitivity to Etoposide-triggered Apoptosis (HL-60) and Enhanced Cell Growth Inhibition or PARP Cleavage (K562). NaB-pretreated HL-60 cells were investigated for their apoptotic response to etoposide relative to untreated controls using the ethidium bromide/acridine orange assay of Cohen and Duke as modified (52). This assay facilitates the microscopic identification of viable and nonviable apoptotic cells and distinguishes apoptotic cells from those that have undergone frank necrosis. HL-60 cells were pretreated for 24 h with 0.4 mM NaB, or sterile water, and then subjected to various concentrations of etoposide for 2 h. Both agents were then washed out, and cells were incubated in drug-free medium for 2 h before processing for staining. As shown in Fig. 5A, NaB pretreatment sensitized cells to the apoptotic effects of 30 and 100 μM etoposide. This phenomenon was characterized further in a time course experiment with 30 μM etoposide (Fig. 5B). At times ranging from 4 to 24 h after drug washout, NaB pre-treatment caused a modest sensitization to etoposide. Taken together, these data indicate that NaB induction of topo II led to an enhancement of the apoptotic efficacy of etoposide in HL-60 cells.

In contrast to HL-60 cells, the erythroleukemia line K562 does not readily undergo apoptosis that can be distinguished morphologically, and its cell death kinetics are delayed in relation to HL-60 cells (18, 53, 56). Therefore, the effect of NaB pretreatment on K562 responsiveness to etoposide was tested in a cell growth inhibition assay. After a 72-h pretreatment with 0.5 mM NaB, the IC50 for a 1-h etoposide exposure was reduced by 3-fold when compared
with control cells (Fig. 6), consistent with the increase in etoposide-mediated DNA damage.

Given that NaB pretreatment conferred etoposide hypersensitivity to K562 cells, the activation of caspase activities was also examined by immunoblotting for cleavage of PARP. Very little PARP cleavage was observed after either the NaB pretreatment alone or with 16 or 24 h of exposure to 30 μM etoposide (Fig. 7). In contrast, NaB pretreatment of K562 cells strongly enhanced the extent of PARP cleavage after these etoposide treatments, as evidenced by the increased intensity of the cleaved immunoreactive band at M, 85,000–89,000.

**Effect of NaB Pretreatment on HL-60 and K562 Cell Cycle Distribution.** Although the NaB enhancement of etoposide cytotoxicity in both leukemia cell lines correlated with topo IIα induction and increased drug-induced protein-linked DNA, the potential contribution of altered cell cycle kinetics to etoposide sensitization was also investigated. topo II-directed drugs are known to be most effective during S-phase but can kill cells to some extent at all phases of the cell cycle (9, 12, 57). Our earlier work demonstrated that a 16-h treatment of HL-60 cells with 0.4 mM NaB could trigger a very transient increase in DNA synthesis (as measured by [3H]thymidine pulse incorporation), which returned to control levels by 24 h (6). Consistent with this earlier finding, the data in Table 1 indicate that 24 h of NaB pretreatment had no substantial effect on HL-60 cell cycle distribution. In fact, the very small but statistically significant increase in G2-M distribution may actually reflect the increased need for topo IIα enzyme activity in chromosomal segregation. Therefore, NaB-mediated etoposide sensitization of HL-60 cells did not appear to result from altered cell cycle kinetics but was rather more likely a result of increased cellular topo IIα content.

The 72-h pretreatment of K562 cells with 0.5 mM NaB caused a modest but significant increase in S-phase distribution, accompanied by a decrease of similar magnitude in

### Table 1  Effect of each NaB treatment on cell cycle distribution of HL-60 and K562 cells

<table>
<thead>
<tr>
<th>Group</th>
<th>G2-M</th>
<th>G0-M</th>
<th>S</th>
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<tbody>
<tr>
<td>HL-60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10.70±0.19</td>
<td>35.68±0.85</td>
<td></td>
</tr>
<tr>
<td>0.4 mM NaB</td>
<td>12.35±0.26</td>
<td>33.75±0.67</td>
<td></td>
</tr>
<tr>
<td>K562</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.91±0.46</td>
<td>51.50±0.98</td>
<td></td>
</tr>
<tr>
<td>0.5 mM NaB</td>
<td>6.86±0.64</td>
<td>60.12±0.82</td>
<td></td>
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* Significant differences from the respective control were determined using Dunnett’s multiple comparison test (p < 0.05).
the G_2–G_1 fraction (Table 1). Although the increased S-phase fraction may contribute in small part to NaB sensitization of K562 cells to etoposide, it is unlikely to account entirely for either the 3-fold decrease in etoposide IC_{50} shown in Fig. 6 or the substantial enhancement of PARP cleavage in Fig. 7. Taken together, these data suggest that the prototypical HDAC inhibitor, NaB, can produce a transient hypersensitivity to the antitumor action of etoposide in experimental leukemia cell lines by inducing functional topo IIα expression with a concordant increase in etoposide-stimulated, enzyme-mediated DNA damage.

**Discussion**

Given that NaB caused a transient increase in HL-60 cell topo IIα expression (6), it was reasoned that a window may exist to maximize rationally differentiation/cytotoxic drug efficacy. In the present report, NaB-induced topo IIα expression correlated with enhanced etoposide-stimulated, protein-linked DNA in two human leukemia cell lines. On the basis of extensive literature demonstrating a positive relationship between topo IIα levels and cytotoxic efficacy of drugs that target this enzyme, the logical assumption was made that increased cleavable complex formation would, in turn, increase etoposide cytotoxicity in NaB-pretreated cells. Indeed, NaB conferred to HL-60 cells a modest hypersensitivity to etoposide-mediated apoptosis, although in K562 cells there was a marked inhibition of cell growth and a substantial enhancement of PARP cleavage.

Other limited attempts to enhance cellular sensitivity to topo II poisons by pharmacological modulation of topo II levels have met with mixed success. For example, substantial induction of topo IIα and β levels was observed in HL-60 cells after 96 h of treatment with all-trans retinoic acid (58). However, induction was not accompanied by increases in etoposide-induced DNA cleavage, and in fact, etoposide-induced apoptosis was reduced significantly relative to controls. In contrast, one clever approach in confluent NIH3T3 cells to block topo IIα transcriptional repression by NF-Y with DNA minor groove binding agents effectively induced the enzyme and caused a dramatic 10–20-fold reduction in the etoposide IC_{50} (35). These investigators argued that manipulation of topo II levels in plateau phase cell populations is more likely to represent situations encountered in vivo than with exponentially growing cultures.

In the present study, a question remains concerning the precise mechanism by which HDAC inhibitors induce topo IIα expression and why there is discord between the magnitude of promoter activation and steady-state enzyme levels. Although our data support a direct relationship between topo IIα promoter activation (from a transfected plasmid construct) and the degree of histone H4 acetylation, there are likely to be promoter-specific consequences of HDAC inhibition that mediate the magnitude, if any, of endogenous gene activation. For example, the high potency HDAC inhibitor, trapoxin, induces only 2% of genes studied (59). Ectopic expression of the histone acetyltransferase P/CAF (as a mimic of NaB treatment) failed to activate topo IIα gene expression in HL-60 cells, suggesting that global activation of histone acetylation is unlikely to account for enhanced topo IIα expression. In other systems, increasing data suggest the cooperation of specific acetylase/deacetylase enzymes with specific transcription factors via corepressor or coactivator proteins (60). For example, in transcriptional activation the yeast GCN5 histone acetyltransferase appears to function in a complex of proteins that bridges activators to the basal transcriptional machinery (61). Conversely, in the case of transcriptional repression, heterodimers of Mad and Max interact with SIN3 corepressor proteins to recruit RPD3 histone deacetylase activity to specific promoters, allowing lysine tails of deacetylated histones to displace activating transcription factors (62). Precisely how histone acetylation status affects the assembly of transcriptional activators and repressors on the endogenous human topo IIα gene promoter remains an ongoing focus of our laboratories.

Other investigators have independently identified additional mechanisms by which HDAC inhibitors may converge on topo IIα regulation. HDAC1 was shown recently to interact directly with topo IIα (63). These investigators also demonstrated that the potent fungal natural product, TSA, could sensitize CCRF-CEM cells and a number of Chinese hamster ovary cell lines to etoposide (63). However, topo IIα levels or activity were not assessed after TSA treatment. Shortly thereafter, Turner’s group independently confirmed the interaction of topo IIα and HDAC1, but these investigators demonstrated paradoxically that TSA treatment confers resistance to etoposide-induced HL-60 cell apoptosis (64). However, this end point was only measured at a single high concentration (100 μM) of etoposide. The present report and these two other recent studies all differ in the type and phasing of HDAC inhibitor treatment and etoposide exposure, making it difficult at present to generalize about the utility of combining these agents. In related work, it should also be noted that TSA was shown recently to induce the activity of the mouse topo IIα gene promoter in mouse 3T3 fibroblasts (65), but this study did not investigate TSA effects on endogenous topo IIα levels or cellular sensitivity to topo II poisons. We have subsequently observed a 3–6-fold induction of the human topo IIα promoter with 24-h exposure to TSA at 100–300 nM in several human tumor cell lines, suggesting that this effect is not limited to the murine gene. Whether TSA behaves in our system with regard to increased enzyme expression and etoposide cellular sensitivity is the subject of ongoing study.

The use of differentiating agents, including NaB, in clinical management of neoplastic disease has shown some promise (66–68), especially when combined with cytotoxic chemotherapy (69). However, enthusiasm has been limited because of the short half-life of fatty acids and a lack of mechanistic rationale for their combination with cytotoxic agents. A search for butyrate analogues possessing a more favorable pharmacokinetic profile than NaB has led to clinical trials with phenylacetate, phenylbutyrate, and tributyryl (70) as

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7 D. J. Kroll, unpublished observation.
8 J. C. Yalowich and D. J. Kroll, manuscript in preparation.
HDAC Inhibitors and topo II Induction

well as pivaloyloxymethyl butyrate (AN9). Newer fungal agents
(TSA, as well as trapoxin and apicidin) with nanomolar inhibi-
tory action on HDAC may also represent useful therapeutics.
However, most clinical work to date has used the aromatic
butyrates. Phenylbutyrate has been used in the treatment of
β-thalassemia because of its ability to induce fetal hemoglo-
bin (71) and in children with urea-cycle disorders (72) be-
cause of its glutamine-scavenging activity. In fact, phenyl-
butyrate was used recently to facilitate the birth of a child
harboring a heterozygous mutation for ornithine transcar-
bamylase (73).

With regard to cancer therapy, others have investigated
HDAC inhibitors for efficacy as single-agent antitumor drugs.
This approach arose originally from the observation that a
number of nonhematopoietic cell lines (including melanoma
and carcinoma of the breast, ovary, lung, prostate, and co-
lon) were responsive to the differentiating effects of butyrate
or butyrate analogues (74–75). A synthetic benzamide HDAC
inhibitor (MS-27–275) also possesses antitumor activity in a
variety of human tumor xenografts in nude mice (76). Simi-
larly, sodium phenylbutyrate can trigger apoptosis in pros-
state carcinoma cells (80). In colon carcinoma, NaB causes
cell cycle arrest by inducing p21 (78), but this effect does not
require p53 (81, 82). The latter finding is provocative in that
the HDAC inhibitors may activate normally p53-dependent
apoptotic machinery in cells lacking functional p53 (83, 84).
Some of the cytostatic effects of phenylacetate and phenyl-
butyrate may also be mediated by their action as ligands for
the peroxisome proliferator-activated receptor γ (85). Al-
though it remains to be demonstrated whether any of these
other cell types also exhibit NaB-inducible topo IIα expres-
sion or whether peroxisome proliferator-activated receptor γ
affects topo IIα expression, HDAC inhibitors are already be-
ing tested in the clinic against several solid tumor types.
Finally, HDAC inhibitors may have antitumor effects that may
only be apparent when in vivo models are used. For example,
TSA was shown recently to act as an angiogenesis inhibitor
in human tumor xenografts of nude mice in part by suppress-
ing hypoxia-responsive tumor suppressor genes (86).

Although our work has focused on the ability of HDAC
inhibitors to influence topo II-dependent drug action, evi-
dence suggests that HDAC inhibitors may also be useful
combination chemotherapy agents via actions independent
of and/or downstream from topo II-mediated DNA damage.
Phenylbutyrate, but not NaB, enhanced doxorubicin cyto-
toxicity in culture, presumably by a loss of numerous cellular
antioxidant defenses (87), although topo IIα levels were not
quantified. In another study (88), pivaloyloxymethylbutyrate
(AN9) was shown to enhance anthracycline efficacy but via
decreased rate of anthracycline aglycone formation and a
reduction in NAD(P)H:quinone oxidoreductase and cyto-
chrome P-450 reductase activities. Oxidative stress attribut-
able to reactive oxygen species has also been implicated in
butyrate sensitization of colorectal carcinoma cells to tumor
necrosis factor-α and Fas ligand (89). Reactive oxygen spe-
cies may also contribute to p53-independent induction of
p21 during growth arrest (83). In addition, NaB potentiates
apoptosis in retinoblastoma cells treated with vincristine or
cisplatin, although the mechanism of this effect was not
eulicated (90). One possible explanation, particularly with
regard to vincristine, is that NaB can cause G2 arrest in
human breast carcinoma cells and drive DNA endorepli-
cation, resulting in polyploidy and delayed cell death (91).

In summary, more detailed understanding of the biological
consequences of altering histone acetylation may lead to
opportunities for synergistic combination chemotherapy be-
tween HDAC inhibitors and classical cytotoxic agents. It
should also be appreciated that HDAC inhibitors possess
other nonhistone activities that may contribute to their sin-
gle-agent and combination antitumor effects, and moreover,
some of these effects may be unique to the HDAC inhibitor
in question. Our current work details a direct link between
HDAC inhibitors, the induction of topo IIα, and the conse-
quent enhancement of etoposide-triggered DNA damage.
However, further studies on HDAC inhibitors in post-DNA
damage processing events may provide insights for maxi-
mizing the antitumor efficacy of these agents.

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References
1. Holm, C., Goto, T., Wang, J. C., and Botstein, D. DNA topoisomerase
2. Holm, C., Stearns, T., and Botstein, D. DNA topoisomerase II must act
at mitosis to prevent nondisjunction and chromosome breakage. Mol.
3. Uemura, T., Ohkura, H., Adachi, Y., Morino, K., Shiozaki, K., and
Yagamida, M. DNA topoisomerase II is required for condensation and
 topoisomerase IIα promoter independently of c-Myb in HL-60 cells. Leuk.
5. Brandt, T. L., Fraser, D. J., Leal, S., Halandras, P. M., Nelson, A. R., and
Kroll, D. J. c-Myb trans-activates the human DNA topoisomerase IIα gene
6. Fraser, D. J., Brandt, T. L., and Kroll, D. J. Topoisomerase IIα promoter
trans-activation early in monocytic differentiation of HL-60 human leukae-
7. Ritke, M. K., Allan, W. P., Fatman, C., Gunduz, N. N., and Yalowich,
J. C. Reduced phosphorylation of topoisomerase II in etoposide-resistant
8. Ritke, M. K., Roberts, D., Allan, W. P., Raymondo, J., Bergoltz, V. V., and
Yalowich, J. C. Altered stability of etoposide-induced topoisomerase II
DNA complexes in resistant human leukemia K562 cells. Br. J. Cancer,
9. Corbett, A. H., and Osheroff, N. When good enzymes go bad: conver-
sion of topoisomerase II to a cellular toxin by antineoplastic drugs. Chem.
drug action: poisoning of mammalian DNA topoisomerase II on DNA by


Molecular Cancer Therapeutics

The Histone Deacetylase Inhibitor Sodium Butyrate Induces DNA Topoisomerase II α Expression and Confers Hypersensitivity to Etoposide in Human Leukemic Cell Lines

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