It’s About Time: Scheduling Alters Effect of Histone Deacetylase Inhibitors on Camptothecin-Treated Cells

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Abstract

Chemotherapeutic treatment with combinations of drugs is front-line therapy for many types of cancer. Combining drugs which target different signaling pathways often lessens adverse side effects while increasing the efficacy of treatment and reducing patient morbidity. A defined scheduling protocol is described by which histone deacetylase inhibitors (HDIs) facilitate the cytotoxic effectiveness of the topoisomerase I inhibitor camptothecin in the killing of tumor cells. Breast and lung cancer cell lines were treated with camptothecin and sodium butyrate (NaB) or suberoylanilide hydroxamic acid on the day of, the day before, or the day after camptothecin addition. Depending on the time of addition, NaB-treated cells displayed a spectrum of responses from protection to sensitization, indicating the critical nature of timing in the use of HDIs. The IC₅₀ (72-hour assay) dose of 100 nmol/L camptothecin could be lowered to 15 nmol/L camptothecin while maintaining or surpassing cell killing of 100 nmol/L camptothecin. Experiments determined that cells arrested in G₂-M by camptothecin were most sensitive hours after camptothecin. Experiments determined that the single agent if combined with an HDI added 24 to 48 hours after camptothecin while maintaining or surpassing cell killing of 100 nmol/L camptothecin could be lowered to 15 nmol/L camptothecin. Therefore, the timing of addition of an HDI to camptothecin-treated cells was examined. This study suggests that HDIs can be used to reduce the toxic effects of camptothecin and facilitate the cytotoxic effectiveness of the topoisomerase I inhibitor camptothecin.

Introduction

Topoisomerase I inhibitors are front-line therapy for advanced liver and lung cancer. Topoisomerase I reduces chromosomal strain during S phase, and its inhibition can lead to the formation of a double-strand break during DNA replication (1–5). Improved camptothecin analogues such as CPT11 (Camptostar or Irinotecan) and Topotecan are being used in clinical settings with more variants being developed and considered for clinical use. Increased clinical interest in camptothecin analogues may increase the demand for methods that limit camptothecin toxicity. While not in clinical use, camptothecin is a representative prototype compound. It shares a mechanism of action with other camptothecin analogues and is well characterized and highly active in cell culture.

Histone deacetylase inhibitors (HDIs) are growing in importance in research and clinical use (6–8). HDIs cause hyperacetylation of histones, relaxing chromatin structure, and activating the transcription of certain regulatory genes, including p21 cip1/waf1 (9). HDIs cause cell cycle arrest and apoptosis through transcriptional activation in some sensitive cell lines (10). Despite promising clinical reports and low toxicity, HDIs have been difficult to use to improve the effectiveness of cancer chemotherapy (7, 8, 11). This may be due in part to the capacity of HDIs to cause a reversible cell cycle arrest (12–14).

There is growing support that adding HDIs to a variety of in vitro treatments, including taxanes (14), all-trans retinoic acid (15, 16), radiation (17), topoisomerase II inhibitors (18), proteosome inhibitors (19), and DNA methylating drugs (20, 21), increases the killing of cancer cells. However, little attention has been paid to the importance of scheduling the addition of an HDI to cytotoxic therapies. This study examines whether the scheduling of HDI and camptothecin alters the effectiveness of in vitro tumor cell cytotoxicity by this combination.

Sodium butyrate (NaB), a short-chain fatty acid with HDI activity, was found to be rapidly metabolized in vivo and thus was difficult to increase to or to maintain at effective therapeutic levels (22). Other compounds with increased specificity for HDACs include trichostatin A (23), the hydroxamic acid analogue LAQ824 (24), and suberoylanilide hydroxamic acid (SAHA; ref. 25). Of these HDIs, SAHA is currently in phase I/II clinical trials (8).

The protein family of inhibitors of apoptosis (IAP) includes XIAP and survivin and is up-regulated in many cancers. Survivin levels are highest during G₂-M (26) when it is phosphorylated by cyclin B/cyclin-dependent kinase 1 (cdk1), which may inhibit its degradation (27). Survivin binds caspase 9, preventing its activation, is associated with the monitoring of mitotic spindles (26–28) and cells with decreased survivin levels are sensitized to topotecan, a hydrophilic camptothecin analogue (29). XIAP inhibits the activation of caspases 3 and 9 and interferes with apoptosis if overexpressed. XIAP and survivin are of particular interest as the protein levels of both are decreased by a variety of HDIs (30–34). Furthermore, HDIs have been shown to decrease cyclin B transcription (35).

Cells with decreased survivin and XIAP are normally more sensitive to camptothecin analogues, but HDIs have previously failed to work optimally in combination with camptothecin. Therefore, the timing of addition of HDI to camptothecin-treated cells was examined. This study suggests that HDIs can be used to increase or prevent cell killing by camptothecin depending on time of addition to the cell culture medium. These data indicate that XIAP and survivin expression may be involved in situations where HDIs increase camptothecin cytotoxicity.
Materials and Methods

Reagents. Camptothecin was a generous gift from Thomas G. Burke, Ph.D. (University of Kentucky, Lexington, KY). NaB was purchased from Sigma-Aldrich (St. Louis, MO). SAHA was purchased from Biovision Research Products (Mountain View, CA). BD ApoAlert Annexin V and Apo 2.7-PE kit and antibodies specific for cyclin B, cdc2/cdk1, were purchased from BD Biosciences (San Diego, CA); p-cdk1 substrate antibody was purchased from Oncogene Research Products (San Diego, CA); β-actin and XIAP antibodies were purchased from Sigma-Aldrich; survivin, poly(ADP-ribose) polymerase (PARP), and phospho-cdk1 (Tyr15) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Sheep anti-mouse and goat anti-rabbit horseradish peroxidase–linked antibodies were purchased from Amersham Life Sciences (Arlington Heights, IL).

Cell culture. The human breast cancer cell lines MDA MB 231 and MDA-MB-435S and the human lung cancer cell line A549 were purchased from the American Type Culture Collection (Manassas, VA). The MDA-MB-435S and MDA MB 231 cell lines were cultured in Eagle's MEM (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 1% MEM nonessential amino acids solution (Life Technologies), and 100 units/mL of penicillin G and 100 μg/mL of streptomycin sulfate (Life Technologies). The A549 cell line was cultured in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum and 100 units/mL of penicillin G and 100 μg/mL of streptomycin sulfate. Cells were grown at 37°C/5% CO2/100% humidity.

Cell cycle analysis. Cells were plated at a density of 5 × 10⁶ per 10 mL in complete medium and were allowed to grow for 24 hours. Cells were then treated with camptothecin at the concentrations indicated in the figure legends and with 5 mmol/L sodium butyrate at the times indicated in the figure legends. Cells were treated with camptothecin for a total of 48 hours. Cells were harvested by combining the cells in the culture medium and the trypsinized monolayer and pelleted by centrifugation. The cell pellet was washed once in PBS, fixed in a 70% solution of ethanol, and DNA content was analyzed as previously described (13). Experiments were done in triplicate, repeated thrice.

Western blot analysis. Cells were plated, treated, and collected as described above, with samples taken 72 or 96 hours after plating, washed once with PBS, counted, and pelleted by centrifugation. Protein extracts were prepared and separated by electrophoresis through 6% or 12% gradient Tris glycine gels (Invitrogen, Carlsbad, CA) as described previously (13). Membranes were probed with antibodies specific to PARP (1:1,000), cdc2/cdk1 (1:2,000), phospho-cdk1 (Tyr15) (1:1,000), cyclin B (1:1,000), survivin (1:1,000), p-cdk1 substrate (1:2,500), XIAP (1:1,000), or β-actin (1:10,000) and the appropriate horseradish peroxidase–linked secondary antibody. Protein bands were visualized using a chemiluminescent enhanced chemiluminescence kit (Amersham Life Sciences) or SuperSignal enhanced chemiluminescent kit (Pierce Biotechnology, Inc., Rockford, IL) as instructed by the manufacturer. At least two protein samples were used, with all blots repeated at least thrice.

Annexin V/propidium iodide fluorescence-activated cell sorting analysis. Cells were plated, treated, and collected as described above; washed once with PBS; and counted and pelleted by centrifugation. Cells were prepared and separated by electrophoresis through 6% or 12% gradient Tris glycine gels (Invitrogen, Carlsbad, CA) as described previously (13). Membranes were probed with antibodies specific to PARP (1:1,000), cdc2/cdk1 (1:2,000), phospho-cdk1 (Tyr15) (1:1,000), cyclin B (1:1,000), survivin (1:1,000), p-cdk1 substrate (1:2,500), XIAP (1:1,000), or β-actin (1:10,000) and the appropriate horseradish peroxidase–linked secondary antibody. Protein bands were visualized using a chemiluminescent enhanced chemiluminescence kit (Amersham Life Sciences) or SuperSignal enhanced chemiluminescent kit (Pierce Biotechnology, Inc., Rockford, IL) as instructed by the manufacturer. At least two protein samples were used, with all blots repeated at least thrice.

Annexin V/propidium iodide fluorescence-activated cell sorting analysis. Cells were plated, treated, and collected as described above; washed once with PBS; and counted and pelleted by centrifugation. Cells were tested for Annexin V binding and propidium iodide (PI) uptake with BD ApoAlert Annexin V and Apo 2.7-PE kit according to the manufacturer's directions with one variation. The amount of Annexin V/FITC was increased from 5 μL per sample to 7.5 μL per sample. Experiments were done in triplicate, repeated thrice.

Cell growth assay. Cells were plated onto 24-well plates at a density of 2 × 10⁴ cells per well for MDA-MB-435S, or 1 × 10⁵ cells per well for A549 and were allowed to grow for 24 hours. Cells were treated with camptothecin at the concentrations indicated in figure legends. Cells were treated with 5 mmol/L sodium butyrate or 10 μmol/L SAHA at the times indicated in figure legends. Cells were treated with camptothecin for a total of 72 hours and were fixed with 15% trichloroacetic acid. Cells were stained with 0.4% sulforhodamine B (SRB) dye in 1% acetic acid for 30 minutes. Excess dye was washed off the plates with 1% acetic acid. Plates were allowed to fully dry. One milliliter of 10 mmol/L Tris (base) was used to release SRB dye bound to cellular protein under gentle shaking for 5 minutes. Two hundred microliters of each sample were added to a 96-well plate, and absorbance was measured at 490 nm. Experiments were done in triplicate, repeated thrice.

Results

NaB decreases net growth in camptothecin-treated cells. Camptothecin analogues with differing blood stability and lipophilicity were previously examined in their ability to promote tumor cell killing (36–38). A range of inhibitors were tested for the capacity to enhance killing of G2-M arrested tumor cells. The HDIs NaB, SAHA, and trichostatin A were found to be effective in increasing camptothecin-mediated tumor cell death.

A combination was initially tested which allowed cells to arrest fully in G2-M with camptothecin (~48 hours) before the addition of the HDI, NaB. To determine the capacity of NaB to potentiate the killing capacity of camptothecin, two cell lines (a) MDA-MB-435S (breast) and (b) A549 (lung) were subjected to a growth assay with combinations of camptothecin and NaB (Fig. 1). Both lines have comparable camptothecin IC50 value of 15 nmol/L and IC80 value of 100 nmol/L. The camptothecin doses listed above were added 24 hours after plating followed 48 hours later with NaB. Cells were harvested 96 hours after plating (72 hours after initial treatment with camptothecin). The two cell lines were more susceptible to the combination treatment than to camptothecin alone. Furthermore, the 15 nmol/L camptothecin dose combined with NaB was as effective at cell killing as treating with 100 nmol/L camptothecin. The breast cancer cell line MDA-MB-231 (camptothecin: IC50 50 nmol/L, IC80 225 nmol/L) was examined and found to have a similar response to the combination of camptothecin and NaB (data not shown).

Timing of histone deacetylase inhibitor addition alters cell growth and affects cell cycle distribution. The initial results did not distinguish if the combination was improved by a defined sequence of addition. To determine whether the scheduling of HDI exposure alters the effectiveness of combining camptothecin and NaB, cells were exposed to 5 mmol/L NaB under four schedules (Fig. 2A). NaB was added at the time of plating (schedule A), 24 hours after plating, and concurrent with camptothecin addition when camptothecin was used (schedule B), 48 hours after plating (schedule C) and 72 hours after plating (schedule D). Cells were fixed 96 hours after plating. Camptothecin and NaB combination treatments were effective in decreasing total cell number compared with camptothecin-treated groups. HDIs cause G0-G1 and G2-M arrest, whereas camptothecin causes cells to arrest only in G2-M. Because both compounds cause cell cycle arrest, it was not known whether decreased cell numbers were due primarily to inhibition of topoisomerase 1 or HDAC, or a combination of the two. The effect of drug treatments on the cell cycle was examined by flow cytometry.

In cells treated with NaB alone, the number of cells in the S phase was greatly decreased. Cells treated with NaB alone accumulated in G2-G1 and G2-M. Cells treated with NaB schedules B and C displayed a profile with more cells in the G2-M (Fig. 2A). Cells treated with 15 nmol/L camptothecin alone arrested in G2-M. When cells treated with 15 nmol/L camptothecin were treated with NaB on schedule A (24 hours before camptothecin), the cells displayed a G0-G1 and G2-M accumulation similar to cells treated with schedule A NaB alone; 15 nmol/L camptothecin + NaB schedule B (concurrent with camptothecin addition) displayed a
mixed profile with a prominent peak of cells in the S-phase DNA content range, some cells in G0-G1 and G2-M.

Camptothecin (15 nmol/L) and NaB schedule C (24 hours after camptothecin addition) displayed a profile similar to that obtained with 15 nmol/L camptothecin also with few cells in G0-G1 and a strong G2-M arrest with declining DNA content. Treatment with 100 nmol/L camptothecin alone was generally toxic to cells with cells showing a degraded DNA content. Schedule A NaB again prevented G2-M arrest by 100 nmol/L camptothecin, with cells accumulating primarily in G0-G1. Camptothecin and schedule B NaB (100 nmol/L) exhibited a population of cells accumulated in the early S phase.

Camptothecin and schedule C NaB (100 nmol/L) produced a profile similar to that obtained by treatment with 100 nmol/L camptothecin alone, with cells seeming to have a progressively degraded DNA content (Fig. 2A). Therefore, NaB addition after camptothecin treatment seemed to enhance camptothecin activity. The decreased cell numbers in schedules A and B is not indicative of increased cell killing, as the number of cells remaining depends on growth rate, growth inhibition, and cell death including apoptosis. Cell cycle analysis indicates that growth arrest plays a major role in determining this assay’s final cell number.

Suberoylanilide hydroxamic acid addition schedule alters cell growth. SAHA is a selective, clinically relevant HDI. The above cell growth experiment was done with SAHA to determine if another HDI could alter the effect of camptothecin activity (Fig. 2B). Cells treated with SAHA alone showed a decrease in cell numbers similar to NaB alone. Populations exposed to SAHA alone or on schedule A or B had fewer cells, due to a reversible G2-G1 and G2-M arrest that is a feature of HDIs. Like NaB treatment, SAHA schedule C was most effective when combined with 15 nmol/L camptothecin (2.71 ± 0.51% cells remaining). Treatment with 100 nmol/L camptothecin followed by SAHA on schedules C (0.81 ± 0.62% cells remaining) and D (0.84 ± 0.25% cells remaining) also exhibited reduced cell number compared with solo agent SAHA schedules C (42.77 ± 5.79% cells remaining) and D (93.33 ± 10.18% cells remaining). The growth inhibition profile was similar between SAHA and NaB-treated cells. This supports the hypothesis that HDAC inhibition influences camptothecin activity. Similar results were obtained with trichostatin A (data not shown).

Long-term survival effects of camptothecin and NaB. Initial findings indicated that cells treated with NaB according to schedules C and D were more sensitive to killing by camptothecin. To test the recovery of cells from various treatments, the growth rates of camptothecin, NaB, and camptothecin + NaB treated cells were compared with an untreated control population over 21 days (Fig. 3). Whereas NaB schedules A and B protect cells from camptothecin (Fig. 2A), it was unknown if camptothecin combined with NaB on any of the tested schedules inhibited growth over a longer period of time. A549 lung cancer cells were treated with 15 and 100 nmol/L camptothecin 24 hours after plating and NaB on schedules A, B, C, and D. Plates were collected on day 3 (72 hours of camptothecin treatment, 96 hours after plating) or fed media free of camptothecin. Plates were then collected or fed every third day until day 21. NaB exposure lasted for 96 continuous hours.

The lung cancer line A549 was chosen for this analysis because of its capacity to recover from camptothecin poisoning in an easily measured time frame. MDA-MB-435S displayed a more prolonged recovery stage, with a lower percentage of cells able to escape camptothecin poisoning. Only about 5% (compared with control) of MDA-MB-435S cells remained after 21 days. The viable cells

Figure 1. Breast and lung tumor cell lines experience increased killing when exposed to a combination of camptothecin (CPT) and NaB. MDA-MB-435S (A) and A549 (B) cells were plated onto 24-well plates. Cells were treated with camptothecin for 72 hours. A549 and MDA-MB-435S cells were treated with 15 and 100 nmol/L camptothecin and/or 5 mmol/L NaB 48 hours after camptothecin treatment, as detailed in the treatment timeline. Cells were fixed 72 hours after camptothecin treatment with trichloroacetic acid, stained with SRB dye, and were evaluated colorimetrically.
Figure 2. Based on the sequence of administration, MDA-MB-435S breast tumor cells show decreased cell growth and cell cycle changes when exposed to a combination of camptothecin (CPT) and HDI. MDA-MB-435S cells were plated onto 24-well plates. Cells were treated as detailed in the treatment timeline with 15 or 100 nmol/L camptothecin for 72 hours. Combination treatment groups were treated with (A) 5 mmol/L NaB or (B) 10 mmol/L SAHA added according to schedules A, B, C, and D. Cells were fixed with trichloroacetic acid 72 hours after camptothecin treatment (96 hours total growth), stained with SRB dye, and were evaluated colorimetrically. Cells were also subjected to fluorescence-activated cell sorting analysis (A) to determine the cell cycle status of camptothecin- and NaB-treated cells. MDA-MB-435S cells were plated onto 100-mm dishes; subjected to camptothecin and NaB schedules A, B, and C treatments as above; and fixed in ethanol 48 hours after camptothecin treatment (72 hours total growth). Cells were then stained with PI and examined via flow cytometry.
seemed clonal. In contrast, A549 reached 50% of control around day 9 when treated with 15 nmol/L camptothecin, and when treated with 100 nmol/L camptothecin, A549 reached 50% of control between days 15 and 18. A549 entered a quiescent phase when the cells became confluent.

Cells treated with NaB alone displayed decreased cell number as measured on days 3 and 6 followed by rapid growth reaching 50% of control on day 15, similar to cells treated with 100 nmol/L camptothecin, which reached 50% of control between days 15 and 16. Both doses of camptothecin combined with NaB schedule D displayed similarly suppressed recovery (data not shown, 15 nmol/L camptothecin + NaB schedule D reached 50% about day 18, 100 nmol/L camptothecin + NaB schedule D reached 50% between days 19 and 20). The delayed recovery of camptothecin + NaB schedules C and D showed the importance of timing with this particular combination therapy, such that the addition of NaB schedules C and D to 15 nmol/L camptothecin increased cell killing to levels achieved with 100 nmol/L camptothecin alone.

**Apoptotic effects of NaB and camptothecin.** To determine if the camptothecin and NaB combination induces apoptosis, MDA-MB-435S cells were treated 24 hours after plating with 15 or 100 nmol/L camptothecin and NaB according to schedules A, B, and C (Fig. 4A). Cysteine proteases referred to as caspases are activated during apoptosis, leading to cleavage of many proteins, including the DNA repair enzyme PARP. PARP is reduced by caspase cleavage from 116 to 85 kDa (39, 40). In samples analyzed at 96 hours (72-hour camptothecin treatment), PARP cleavage increased in the 15 nmol/L camptothecin + NaB schedule C–treated cells. Treatment of the cells with 15 nmol/L camptothecin + NaB schedule C resulted in the appearance of more PARP cleavage product than treatment with 100 nmol/L camptothecin alone. There was some PARP cleavage observed in the solo treatments 15 nmol/L camptothecin and NaB schedules B and C. Overall levels of PARP protein were significantly decreased in the 100 nmol/L camptothecin with schedule C HDI protocol and a slight reduction of total PARP protein was observed with the schedule B HDI protocol. The 85-kDa cleavage product was not detected in 100 nmol/L with NaB.
schedule C at 96 hours. In cells treated with this dose of camptothecin, cells may be dying by a caspase independent means or have passed a point in apoptosis where the cleavage product can be detected.

To examine cell death induced by camptothecin and NaB, cells were treated with 15 and 100 nmol/L camptothecin and NaB on schedules A, B, and C. Apoptosis was measured using Annexin V/PI (Table 1), an assay that differentiates among viable, early apoptotic, and late apoptotic/necrotic cells.

Cells treated with camptothecin at either 15 or 100 nmol/L and NaB schedule C displayed a decrease in viable cells and an increase in early apoptotic and late apoptotic/necrotic populations. Cells that were treated with either dose of camptothecin and NaB schedule A or B did not display similar decreases in viable cells compared with CPT as a solo agent. The protections previously observed in cell cycle analysis are reflected in the ratio of viable cells compared with the apoptotic classifications.

**NaB/camptothecin timing affects inhibitors of apoptosis.**

Cells arrested by camptothecin often exhibit high levels of cyclin B and cdk1 (41). Because an active cyclin B/cdk1 complex is responsible for maintaining survivin levels in G2-M arrested cells (27, 42), levels of cyclin B, survivin, cdk1, and inhibitory phosphorylation state of cdk1 were examined (Fig. 5 A). Cells treated with NaB alone on schedule C exhibited levels of these proteins similar to those found in control cells. Whereas both NaB schedules B and C had populations of cells in G2-M, NaB schedule B–treated cells had been under HDI treatment for 24 hours longer than NaB schedule C–treated cells. HDIs have been shown to decrease levels of cyclin B during G2-M (35, 43, 44); thus, the longer exposure to HDI on schedule B may account for the lower expression level of cyclin B. Cells treated with camptothecin and NaB schedules A and B did not display an increase in cyclin B, cdk1, p-cdk1, or survivin. This was an expected result, as these cells were mainly arrested at stages that inhibit the full effectiveness of camptothecin treatment (Fig. 2).

The combinations most effective in causing cell death, 15 nmol/L camptothecin + NaB schedule C and 100 nmol/L camptothecin + NaB schedule C exhibited cyclin B and survivin levels that were decreased compared with cells treated with camptothecin alone. Cells treated with 15 nmol/L camptothecin and NaB schedule C displayed decreased levels of cdk1 compared with 15 nmol/L camptothecin alone, whereas the addition of NaB schedule C to 100 nmol/L camptothecin did not appreciably alter the levels of cdk1. There was some variation in results of cdk1 and p-cdk1 levels between cells treated with 100 nmol/L camptothecin and those treated with 100 nmol/L camptothecin and NaB schedule C.

**Figure 4.** Timing of NaB addition in relation to camptothecin (CPT) significantly alters apoptosis. A, PARP cleavage is increased by the addition of NaB 24 hours after camptothecin. MDA-MB-435S cells were treated with camptothecin 24 hours after plating, with NaB added according to schedules A, B, and C, as detailed in the treatment timeline. Ninety-six hours after plating (72 hours after camptothecin treatment), cells were harvested and lysed with lysis buffer (dilution of 5 × 10⁶ cells/μL). Lysate equivalent to 5 × 10⁴ cells was added to each well. Blots were probed with HrPA-linked antibodies. B, camptothecin and NaB schedule C decrease viable population and increase apoptotic/necrotic fractions, as detected by exposure of phosphatidylserine and PI uptake. Cells plated and treated as per Western blotting procedure. Ninety-six hours after plating (72 hours after camptothecin treatment), cells were harvested and labeled with FITC/Annexin V and PI and examined by fluorescence-activated cell sorting. Cell debris (Annexin V−, PI+) makes up the remaining percentage.
Camptothecin (15 nmol/L) with NaB schedule C displayed a small decrease in p-cdk1 levels. The presence of less cyclin B in these treatment groups would result in less overall kinase complex, which could affect phosphorylation of target proteins such as survivin.

To determine possible changes in cdk1 substrate phosphorylation, cells were treated with NaB on schedule C, with and without camptothecin at either 15 nmol/L camptothecin or 100 nmol/L camptothecin (Fig. 5B). Increased phosphorylation of cdk1 substrates was seen in cells treated with NaB C alone. When camptothecin was combined with NaB schedule C, the level of phosphorylated substrate decreased. Decreased cyclin B/cdk1 complex activity would allow survivin to be degraded, because survivin stability has been shown to be affected by phosphorylation at Thr34 by cyclin B/cdk1 (27). Lower survivin levels in camptothecin + NaB schedule C treatment groups were observed. Decreased survivin levels may contribute to cell killing. Survivin binds and sequesters caspases and is important to cells arrested in G2-M for prevention of apoptosis.

XIAP levels in camptothecin or camptothecin + NaB schedule C treated cells were examined (Fig. 5B). Schedules A and B were not

### Table 1. NaB scheduling changes viable fraction of camptothecin-treated cells, as detected by exposure of phosphatidylserine and PI uptake

<table>
<thead>
<tr>
<th>Schedule</th>
<th>15 nmol/L camptothecin</th>
<th>100 nmol/L camptothecin</th>
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<tbody>
<tr>
<td>NaB (-)</td>
<td>V  92.27</td>
<td>EA 1.90</td>
</tr>
<tr>
<td>NaB A</td>
<td>V 80.24</td>
<td>EA 6.61</td>
</tr>
<tr>
<td>NaB B</td>
<td>V 74.74</td>
<td>EA 6.62</td>
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<tr>
<td>NaB C</td>
<td>V 83.86</td>
<td>EA 3.31</td>
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NOTE: Cells plated and treated as per Western blotting procedure. Forty-eight and 72 h after camptothecin treatment, cells were harvested and labeled with FITC/Annexin V and PI, and examined by fluorescence-activated cell sorting. Results are expressed as percentages, and cell debris (Annexin V+, PI+) makes up the remaining percentage.

Abbreviations: V, viable; EA, early apoptosis; LA/N, late apoptosis/necrosis.

Figure 5. Camptothecin (CPT) treatment followed by NaB decreases both (A) survivin and associated regulatory proteins and (B) cdk1 substrate phosphorylation and XIAP levels. MDA-MB-435S cells were treated with camptothecin 24 hours after plating, with NaB added according to schedules A, B, and C as detailed in the treatment timeline. Forty-eight hours after camptothecin treatment, cells were harvested and lysed with lysis buffer (dilution of 5 x 10^6 cells/μL). Lysate equivalent to 5 x 10^4 cells was added to each well. Blots were probed with HRPAlinked antibodies.
used in this experiment, because these HDI schedules were ineffective in improving cytotoxicity. Camptothecin-treated (15 and 100 nmol/L) cells were observed to increase levels of XIAP. The addition of NaB schedule C to camptothecin-treated cells decreased the levels of XIAP. As XIAP prevents the activation of caspases 3 and 9, cells treated with this combination of drugs were more likely to undergo apoptotic cell death.

**Discussion**

These studies show that the augmentation of camptothecin-induced cell death by HDIs is most effective when HDIs are added to cells already arrested in G2-M by camptothecin. HDIs added before or at the same time as camptothecin limit the number of cells affected by camptothecin. Of particular importance to the clinician is the potential use of HDIs to achieve a 7-fold increase in the effectiveness of an IC50 dose of camptothecin to a level similar to an IC80 dose in both 3-day killing and long-term recovery. This increase in effectiveness could allow a patient to potentially receive lower levels of camptothecin analogues resulting in a similar treatment outcome with reduced toxicity to the patient.

Johnson et al. and Kim et al. (8) previously reported on combinations of HDIs and topoisomerase II inhibitors and included camptothecin as a negative control. Johnson et al. found that cells treated with camptothecin followed by treatment with trichostatin A 30 minutes later did not significantly increase killing (45). In addition, Kim et al. found nonsignificant variations in killing when time of addition of camptothecin was varied by 4 hours before or after addition of trichostatin A. Furthermore, Kim et al. examined a wide variety of topoisomerase II inhibiting compounds, all of which resulted in increased cell killing when combined with HDIs (18).

The limitations on the effect of HDIs on topoisomerase I inhibitors, as tested by Johnson et al. and Kim et al., might have been due to the ability of HDIs to cause a reversible G0-G1 or G2-M arrest. Cells arrested by HDIs would not enter the S phase and would therefore be protected against camptothecin, an S phase-specific drug. However, this does not explain why the topoisomerase II inhibitors tested by Johnson et al. and Kim et al., in which camptothecin was used as a control, displayed increased cytotoxic activity.

Topoisomerase II inhibitors are effective during S and G2-M phases. HDIs have been shown to increase the transcription of the topoisomerase IIα subunit during the G0-G1 phase, possibly extending the cytotoxic activity of topoisomerase II inhibitors to the G0-G1 phase (46, 47). Direct protein-protein interactions between topoisomerase II and HDAC1 and HDAC2 have also been reported (45, 48). Because of the wide variety of transcriptional changes caused by HDIs, compounds with different mechanisms of action may interact with HDIs by separate pathways.

Timing is particularly crucial to the effective use of HDIs with topoisomerase I poisons, which are only active in the S phase. Johnson et al. and Kim et al. (these studies added HDI and camptothecin concurrently) did not observe increased killing by camptothecin; it was therefore reasoned that allowing camptothecin to act during the S phase, causing a G2-M arrest followed by HDI treatment, might be a more effective strategy than using camptothecin as a solo agent. Arresting cells with camptothecin in G2-M followed by exposing cells to an HDI caused increased apoptosis and cell death (Figs. 2 and 4) as well as extended the time to recovery (Fig. 3).

The data presented in this study are consistent with the findings of Johnson et al. and Kim et al. in that cells treated with HDI before or simultaneously with camptothecin do not display greatly increased killing over the HDI alone. Indeed, cytostatic levels of staurosporine that causes G0-G1 arrest protect against camptothecin levels 100× higher than LD100 (49). A similar form of protection using HDI was found, although the upper limits of camptothecin protection were not tested.

When cells were treated with camptothecin, a G2-M phase arrest was observed, concurrent with an increased level of cyclin B, cdk1, and survivin expression. These changes were often observed in G2-M arrested cells treated with certain chemotherapeutic reagents (3, 41, 50). Cells treated with camptothecin followed by HDI treatment schedule C or D (24 and 48 hours after camptothecin) had inhibited long-term growth compared with either camptothecin or HDI alone. Camptothecin treatment combined with HDI schedule C displayed increased apoptosis. Cells also exhibited decreased cyclin B and cdk1 substrate phosphorylation when NaB schedule C was used (24 hours after camptothecin; Fig. 5).

HDIs as single treatment agents have been shown to decrease cyclin B transcription (43) which may be the mechanism by which cyclin B and cdk1 substrate phosphorylation are decreased in camptothecin and HDI schedule C–treated cells. Cyclin B levels may also be decreased due to turnover of cyclin B mRNA (51) or selective breakdown of cyclin B (52, 53). Reduction of cyclin B/ cdk1 protein levels and cdk1 substrate phosphorylation may account for the observed decrease in survivin levels (27). Therefore, timing of HDI addition could have acted to sensitize or protect against camptothecin. Furthermore, the decrease of cyclin B and cdk1 activity is a growth signal associated with cells leaving G2-M (54).

The camptothecin + NaB schedule C used in these experiments caused increased apoptosis as determined by cleavage of PARP, fluorescence-activated cell sorting analysis, and a decrease in survivin and XIAP protein levels. Survivin has recently been shown to be important for cells escaping camptothecin poisoning (29, 55), as well as to regulate the mitotic spindle assembly checkpoint (26). XIAP, a relative of survivin, was found to be up-regulated in camptothecin-treated cells, with the subsequent addition of HDI causing XIAP levels to decrease. The changes in survivin and XIAP regulation may be associated with increased cell death through a lack of caspase inhibition or from disruption of the spindle assembly checkpoint during mitosis.

It is important to note that cells treated with NaB schedules A and B alone did not increase cdk1, cyclin B, or survivin, changes associated with camptothecin treatment. Cells treated with NaB schedule C alone did display levels of cdk1, cyclin B, or survivin comparable with control cells but higher than that seen in NaB schedule A or B–treated cells. When NaB was added along with 15 nmol/L camptothecin or 100 nmol/L camptothecin on schedules A (24 hours before camptothecin) and B (at the same time as camptothecin), increases in the protein levels of cyclin B, cdk1, and survivin were not observed. This suggests that a majority of cells were protected against the effects of camptothecin by inhibition of cell cycle progression by HDIs.

Combining the data presented herein, a possible mechanism of killing for this combination therapy may be proposed. This scenario emphasizes the importance of adding an HDI after tumor cells have been arrested in G2-M by a topoisomerase I
inhibitor. HDIs have been reported to decrease XIAP and cyclin B, which can lead to the depletion of survivin. In G2-M arrested cells, this loss of caspase inhibition would lead to the observed increase in apoptosis.

In addition to a loss of caspase activation, other events may contribute to the enhanced killing of cells treated with camptothecin and HDI. Understanding how HDIs work in combination with other drugs is essential to increasing their effectiveness in the clinical setting. The results of this study highlight the need to evaluate the role of scheduling in using HDIs with topoisomerase I inhibitors. In this context, the molecular pathways affected by the combination of camptothecins and HDIs, the mechanism behind enhanced tumor cell killing and the possible influence of aberrant growth signals initiated by HDIs resulting in the premature reduction of cyclin B/cdk1 levels are being investigated.

References


It's About Time: Scheduling Alters Effect of Histone Deacetylase Inhibitors on Camptothecin-Treated Cells

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