Synergistic antitumor effect between vorinostat and topotecan in small cell lung cancer cells is mediated by generation of reactive oxygen species and DNA damage-induced apoptosis

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Abstract
The topoisomerase-I (topo-I) inhibitor topotecan, derivative of camptothecin, is the only registered drug for relapsed small cell lung cancer (SCLC). The histone deacetylase inhibitor vorinostat has shown preclinical and clinical antitumor activities in hematologic malignancies and solid tumors, including SCLC, and has recently been approved for the treatment of cutaneous T-cell lymphomas. In this study, we analyzed the antitumor effect of vorinostat combined with topotecan or camptothecin in topo-I inhibitor-sensitive H209 and inhibitor-resistant H526 SCLC cells. Simultaneous or sequential exposure (24 h delay) to either agent resulted in strong synergistic cytotoxic effect in both cell lines, as shown by calculating combination index, and confirmed by growth in soft agar. Combination treatments increased S-phase cell cycle arrest paralleled by apoptosis as measured by hypodiploid peak formation, Annexin V binding, DNA fragmentation, and mitochondria destruction. The apoptotic process was triggered by a caspase-dependent mechanism and can be ascribed to the phosphorylation of H2AX, a reporter of DNA double-strand breaks. These effects were paralleled by an increase of topo-I/DNA covalent complexes induced by combination treatment and suggest a potentiation by vorinostat of topotecan-induced DNA damage. Finally, oxidative injury played a significant functional role in the observed enhanced lethality because coadministration of the antioxidant α-acetyl-L-cysteine blocked reactive oxygen species generation, apoptosis, and mitochondria destruction induced by the vorinostat/topotecan combination. To our knowledge, this is the first demonstration of a synergistic antitumor effect between topotecan and vorinostatin SCLC. Because no well-established treatment is available for recurrent SCLC patients, our results indicate that this drug combination should be explored clinically. [Mol Cancer Ther 2009;8(11):3075–87]

Introduction
Small cell lung cancer (SCLC) is an aggressive form of cancer, which accounts for ∼12% to 16% of lung neoplasms (1). Although good response rates have been achieved using combination chemotherapy with cisplatin and etoposide as the most widely used regimen, only a small proportion of patients survive after 5 years (2). Most patients relapse within 1 year of starting first-line treatment due mainly to intrinsic or acquired resistance to chemotherapy. Recently, several new chemotherapeutics as well as targeted agents have been tested in SCLC; however, only minor positive results were obtained (2). Thus, alternative agents and new integrated therapeutics are needed.

Histone deacetylase inhibitors (HDACI) represent a new class of anticancer agents that, enhancing the acetylation of core histones, weaken histone-DNA interactions and consequently increase DNA accessibility and activate transcription (3, 4). These compounds act very selectively to alter the transcription of <2% to 5% of expressed genes (3, 5) and induce tumor cell growth arrest, differentiation, and apoptosis by mechanisms that cannot be solely attributed to the level of histone acetylation (6). In addition to histones, reversible acetylation of other proteins such as p53, α-tubulin, and heat shock protein 90, has also been described (7). Several HDACIs exhibit antitumor effects in preclinical animal models at amounts that have little or no toxicity (3), and some are in advanced clinical studies either as single agents or in combination with conventional chemotherapy or biologicals (3, 4, 8–10). One of the most promising HDACIs, vorinostat (suberoylanilide hydroxamic acid), has shown significant preclinical and clinical antitumor activities in both hematologic malignancies and solid tumors...
including SCLC and represents the first HDACi to be recently approved by the Food and Drug Administration for the treatment of cutaneous T-cell lymphoma with progressive, persistent, or recurrent disease during or following two systemic therapies (3, 9–11).

The nuclear topoisomerase-I (topo-I) and topo-II enzymes are responsible for regulating and maintaining DNA topology. In particular, by breaking and religating DNA strands, they relax DNA supercoiling generated by transcription, replication, and chromatin remodeling (12). Topoisomerase enzymes are targeted by several inhibitors currently used as antitumor agents. Several classes of these agents including topo-I inhibitors interfere with the reversible topo-DNA interaction by covalently trapping the enzyme on the DNA and inducing irreversible DNA damage during the replication process (13). It has been suggested that increased accessibility of the topoisomerase enzymes to chromatin DNA could result in increased sensitivity to the inhibitors (14, 15). In this regard, relaxation of chromatin structure induced by HDACIs may facilitate accessibility of the topoisomerase enzymes to DNA and increase the cytotoxicity of topoisomerase inhibitors.

In this study, we analyzed the antitumor effect of the HDACI vorinostat in combination with the topo-I inhibitors camptothecin and its water-soluble semisynthetic derivative topotecan, the only registered drug for second-line therapy in relapsed SCLC (16). Topotecan has shown antitumor activity in both chemosensitive and chemoresistant SCLC and has a nonoverlapping toxicity profile with other agents used in the treatment of SCLC (16).

We showed that vorinostat in combination with either camptothecin or topotecan exerted a strong synergistic cytotoxic effect in SCLC cells, including those resistant to topo-I inhibitors. This effect was not dependent on the schedule of treatment and was paralleled by a late S-phase cell cycle arrest and pronounced apoptosis triggered by a caspase-dependent mechanism. The induction of phosphorylated H2AX (γH2AX), which is a marker of DNA damage, the generation of reactive oxygen species (ROS), and the destruction of mitochondrial membrane were observed only in cells treated by the vorinostat/topotecan combination and were paralleled by an increase of topo-I/DNA covalent complexes, suggesting a potential mechanism for the observed synergistic antitumor interaction.

**Materials and Methods**

**Materials**

Clinical-grade vorinostat was provided by Merck & Co. Stock solutions were prepared in DMSO and diluted to appropriate concentrations in culture medium before addition to the cells. Topotecan (Hycamtin) was supplied by GlaxoSmithKline and camptothecin was obtained from Alexis Biochemicals. Antibodies were purchased as follows: acetylated histone H3 and γH2AX from Upstate, poly(ADP-ribose) polymerase (PARP) from BD Biosciences, caspase-3 from Cell Signaling Technology, and topo-I, γ-tubulin, and p53 from Santa Cruz Biotechnology. MTT was from ICN Biomedicals. Enhanced chemiluminescence immunodetection reagents were from GE Healthcare. All media, serum, antibiotics, and glutamine were from Cambrex Biosciences and grown in suspension in RPMI supplemented with 10% heat-inactivated fetal bovine serum, penicillin (50 units/mL), streptomycin (500 μg/mL), and 4 mmol/L glutamine in a humidified atmosphere of 95% air and 5% CO2 at 37°C. Cell viability was measured after treatment (72 or 96 h) in 96-well plates by an MTT assay as described previously (17).

**Drug Combination Studies**

Drug combination studies were based on concentration-effect curves generated as plots of the fraction of unaffected (surviving) cells versus drug concentration after 72 or 96 h (in the case of sequential experiments) of treatment. To explore the relative contribution of each agent to the synergism, serial dilutions of doses of the two agents (vorinostat/topotecan or camptothecin) in combination were tested with different molar ratios: equi-active doses (50:50 cytotoxic ratio) of the two agents (IC50), higher relative doses of topotecan or camptothecin (25:75 cytotoxic ratio; IC25 of vorinostat/IC75 of topotecan or camptothecin), and higher relative doses of vorinostat (IC75 of vorinostat/IC25 of topotecan or camptothecin).

For sequential combinations, serial dilutions of equi-active doses were tested in three different sequences of treatment: simultaneous (both drugs were given together 24 h after seeding and cell growth assessment was done after 96 h) or when each one was applied 24 h before the other (vorinostat or topotecan or camptothecin was added 24 h after seeding followed by the indicated combined drug after 24 h, and cell growth assessment was done after 72 h), so that cells were exposed for 96 h to the first applied drug and for 72 h to the following combined agent. Synergism, additivity, or antagonism was quantified by determining the combination index (CI) calculated by the Chou-Talalay equation (18) as described elsewhere (19) and by using the software CalcuSyn (Biosoft). Assuming 0.8 as the cutoff, CI < 0.8, CI = 0.8–1, and CI > 1 indicate synergistic, additive, and antagonistic effects, respectively. The dose reduction index (DRI) represents the measure of how much the dose of each drug in a synergistic combination may be reduced at a given effect level compared with the dose of each drug alone. The linear correlation coefficient (r) of the median-effect plot was always between 0.90 and 0.99, indicating good conformity of the data (18).

**Analysis of Cell Cycle Kinetics**

Analysis of cell cycle kinetic was done at the indicated times as described previously (20). Briefly, cells were harvested, fixed in 70% ethanol, and stored at -20°C until analysis. After nuclear DNA staining with propidium iodide, flow cytometry was done in duplicate by a FACScan flow cytometer.
cytometer (Becton Dickinson). For each sample, 20,000 events were stored and cell cycle analysis was done by the ModFit LT software (Verity Software House). FL2 area versus FL2 width gating was done to exclude doublets from the G2-M region. The percentage of apoptotic cells was calculated in the subdiploid region of the DNA content, registered as FL2 signals in linear scale. To avoid cell debris contamination due to necrotic cell death, cells were selected by side scatter versus DNA signals (FL2) gating.

**Soft-Agar Clonogenic Assay**

The soft-agar clonogenic assay was done as reported previously (21). In brief, cells were plated at 2 × 10^3/mL in an upper layer of 0.3% bacto-agar in RPMI (1:1) and 20% fetal bovine serum over an underlayer of 0.8% agar in RPMI in 24-well plates, and after 3 h, the cells were treated with vorinostat at doses of 1/5 and 1/10 of the in vitro IC50 and/or toposcan at doses of 1/6 and 1/12 of the in vitro IC50. Cultures were maintained for 3 weeks in a dry atmosphere of 95% air and 5% CO2 at 37°C. Cellular colonies were then stained with 150 μL nitroblue tetrazolium and colonies >50 μm were scored with an Artek 880 colony counter (Arték). The data were analyzed by Image-Pro Plus software.

**Protein Extraction and Western Blotting**

Cells grown and treated as indicated were collected, lysed, and separated on SDS-PAGE, and proteins were transferred to nitrocellulose paper, immunoblotted with specific antibodies, and probed with the appropriate horse-radish peroxidase–linked IgG as described elsewhere (19). Immunoreactive bands were detected by enhanced chemiluminescence. For the immunodetection of topo-I, nuclear extracts were prepared as described previously (22).

**Apoptosis Assays**

Annexin V binding was identified by flow cytometry using Annexin V-FITC staining following the manufacturer’s instructions (Becton Dickinson). Internucleosomal DNA fragmentation (ladder) was measured after extraction of low molecular weight DNA, PAGE, and silver staining as reported elsewhere (23). Briefly, 1 × 10^7 cells were resuspended in Tris-EDTA and lysed in 20% SDS and DNA was precipitated in 2 mol/L NaCl. The high molecular weight fraction was sedimented by high-speed centrifugation, and fragmented DNA was extracted from the aqueous phase with phenol and chloroform and then precipitated with ethanol. After resuspension in loading buffer, DNA was electrophoresed using 10% polyacrylamide gel and visualized by silver staining.

**Measurement of ROS Generation**

ROS generation was analyzed by flow cytometry using the ROS-sensitive dye hydroethidine as described previously (24). Briefly, the cells, untreated or treated as indicated, were incubated with 20 ng/mL hydroethidine for 1 h at 37°C in the dark and then trypsinized from the culture dishes and re-suspended in PBS. ROS generation was measured as significant increases in fluorescence intensity within 45 min by CellQuest data analysis software.

**Mitochondrial Potential Analysis**

Mitochondrial functions such as mitochondrial membrane potential can be studied in live cells using vital lipophilic cationic dyes. We took advantage of the MitoTracker Red CMXROS fluorescent dye (Invitrogen), a specific mitochondria dye derived from X-rosamine that exhibits good photostability and is usually applied to analyze changes in mitochondrial membrane potential in fixed cells by confocal microscopy. We evaluated CMXROS staining of live cells by flow cytometry, which has been reported to be a more quantitative method to measure mitochondrial membrane potential (25). Cells were treated with the drugs at indicated concentrations and collected after 24, 48, and 72 h and then harvested, centrifuged, and washed with PBS. Cell suspensions were incubated in 1 mL PBS with 500 nmol/L CMXROS dye for 15 to 45 min in the dark at 37°C. After incubation, the cells were immediately analyzed. The transition of mitochondrial from a polarized to depolarized status results in leakage of dye and a consequent decrease of red fluorescence as measured by CellQuest data analysis software as described previously (26, 27).

**DNA Relaxation Assays**

Nuclear extracts, prepared as described before (22), were incubated in 20 μL reaction volume containing 0.5 μg negatively supercoiled pBlueScript KSII (+) DNA and reaction buffer as described previously (28); to assess the effects of vorinostat on enzyme activity, increasing concentrations of nuclear proteins were added to the mixture. Reactions were stopped with a final concentration of 0.5% SDS after 1 h at 37°C. The samples were electrophoresed in a horizontal 1% agarose gel in 50 mmol/L Tris, 45 mmol/L boric acid, and 1 mmol/L EDTA. The gel was stained with ethidium bromide (5 μg/mL) and destained with water and the image was acquired under UV illumination by ChemiDoc XRS Bio-Rad). Where indicated, purified topo-I (28) and vorinostat, or camptothecin, where incubated at 37°C in the reaction mixture with DNA substrate.

**Detection of Covalent Topo-I/DNA Complexes**

Topo-I/DNA adducts were isolated using an Immuno Complex Enzyme bioassay as described previously (29). Briefly, 1 × 10^7 treated and untreated cells were pelleted and immediately lysed with 1 mL of 1% sarkosyl. After syringe-drawing, cell lysates were gently layered on step gradients containing four different cesium chloride solutions (2 mL of each) of following densities: 1.82, 1.72, 1.50, and 1.45 (30). Tubes were centrifuged at 31,000 rpm for 21 h at 20°C. Half-milliliter fractions were collected from the bottom of the tubes and the DNA peak was localized by determining the absorbance at 260 nm. In all, 5 μg DNA was mixed with an equal volume of 25 mmol/L sodium phosphate (pH 6.5) and applied to nitrocellulose paper using a slot-blot vacuum manifold (Bio-Rad). The membranes were equilibrated in TBST [10 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, and 0.05% Tween 20] and then incubated overnight at 4°C with topo-I antibody diluted in TBST containing 5% nonfat dried milk.

**Statistical Analysis**

All determinations were done in quadruplicates or triplicates as indicated and each experiment was repeated at least three times. Representative results from flow cytometry analysis, Western blotting, DNA ladder analysis, DNA
Results

Vorinostat and Topo-I Inhibitors Interact Synergistically to Induce a Cytotoxic Effect in SCLC Cells

In several in vitro cancer models, HDACIs in combination with drugs targeting DNA induce a synergistic inhibition of cell viability as well as apoptosis (4, 31). To evaluate the potential synergistic effect of vorinostat in combination with the topo-I inhibitors topotecan or camptothecin in SCLC cell lines, H209 (wild-type p53) and H526 (mutant p53) cells were exposed for 72 h to increasing concentrations of the drugs, alone or in combination (vorinostat/topotecan or vorinostat/camptothecin), and cell viability was evaluated by the MTT assay. Vorinostat reduced the viability of both H209 and H526 cells at micromolar concentrations (Fig. 1A) and induced a time-dependent histone H3 acetylation in both cell lines (Fig. 1A, inset). In contrast, the mutant p53 H526 cell line appeared strongly resistant to both topotecan and camptothecin, with respective IC50 values of ∼1,000- and 160-fold higher than those observed in wild-type p53 H209 cells (Fig. 1B and C).

The interactions between vorinostat and either topotecan or camptothecin appeared to be strongly synergistic as shown by a median drug effect analysis calculating CIs in both cell lines (Table 1). In details, to explore the relative contribution of each agent to the synergism, different vorinostat/topotecan or vorinostat/camptothecin combinations were compared by testing equipotent doses of the two agents (50:50 cytotoxic ratio), higher relative doses of vorinostat (75:25 cytotoxic ratio), or higher relative doses of topotecan or camptothecin (25:75 cytotoxic ratio). A strong synergistic effect with low CIs (CIs < 0.8) was shown for both vorinostat/topotecan and vorinostat/camptothecin combinations when equipotent doses of the two drugs were used (50:50 cytotoxic ratio) in H209 cells. The same results were observed in the H526 cell line resistant to both topotecan and camptothecin (see Fig. 1B and C). Similar or even stronger effects were observed in both cell lines when lower doses of vorinostat were used (25:75 cytotoxic ratio). In contrast, when higher doses of vorinostat were combined with lower doses of either camptothecin or topotecan (75:25 cytotoxic ratio), a synergistic effect was observed only for the vorinostat/topotecan combination in H209 cells, whereas an additive or antagonistic effect was observed for the other combinations.

Furthermore, we explored different schedules of treatment combining equitoxic concentrations of vorinostat.

Figure 1. Effects of vorinostat, topotecan, and camptothecin on the viability of H209 and H526 SCLC cell lines and acetylation of histone H3 induced by vorinostat. Approximately 6 × 10^3 H526 or 12 × 10^3 H209 cells per well were cultured in 96-well plates and incubated with the indicated doses of vorinostat (A), topotecan (B), and camptothecin (CPT; C) for 72 h. Cell viability, assessed by the MTT method, is expressed as percentage of control for each time point. Mean ± SD from at least three independent experiments done in quadruplicates. Acetylated histone H3 (AcH3) protein expression was analyzed by Western blotting in H526 and H209 cells treated with vorinostat at a concentration corresponding to IC50 72 h (3 and 4 pmol/L, respectively) and harvested at the indicated times (A, inset).
and topotecan in both H526 and H209 cell lines. As shown in Table 1, a sequential exposure with 24 h delay to the two agents in combination produced a strong synergistic cytotoxic effect similar to that observed for simultaneous treatment, indicating that the synergistic interaction between vorinostat and topotecan was not schedule-dependent. Interestingly, the evaluation of DRI values, which represent the order of magnitude (fold) of dose reduction, obtained for the IC_{50} (DRI_{50}) in combination treatment compared with single-drug treatment, showed fold reductions of 4 to 9.4 for vorinostat and 3.6 to 15.88 for topotecan in all three sequential combination schedules described earlier (data not shown).

**Table 1. CI and DRI values according to the different cytotoxic ratios of vorinostat/topotecan and vorinostat/camptothecin or to the different schedules of exposure to vorinostat/topotecan combinations**

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Vorinostat + topotecan</th>
<th>Vorinostat → topotecan</th>
<th>Topotecan → vorinostat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CI_{50} ± SD*</td>
<td>DRI_{50} ± SD†</td>
<td>CI_{50} ± SD*</td>
</tr>
<tr>
<td></td>
<td>Vorinostat Topotecan</td>
<td>Vorinostat Topotecan</td>
<td>Vorinostat Topotecan</td>
</tr>
<tr>
<td>H209</td>
<td>0.38 (0.04) 4.5 (1.5)</td>
<td>6.94 (0.28)</td>
<td>0.28 (0.015) 7.44 (0.9)</td>
</tr>
<tr>
<td>H526</td>
<td>0.39 (0.05) 4.46 (1.07)</td>
<td>5.99 (1.4)</td>
<td>0.41 (0.064) 9.4 (0.8)</td>
</tr>
</tbody>
</table>

NOTE: 50:50, serial dilutions from vorinostat IC_{50} [4 μmol/L (H209) and 3 μmol/L (H526)]/topotecan IC_{50} [0.07 μmol/L (H209) and 4 μmol/L (H526)] or camptothecin IC_{50} [0.008 μmol/L (H209) and 1.5 μmol/L (H526)]; 75:25, vorinostat IC_{50} [10 μmol/L (H209) and 20 μmol/L (H526)]/topotecan IC_{25} [0.001 μmol/L (H209) and 0.1 μmol/L (H526)]; and 25:75, vorinostat IC_{25} [1.15 μmol/L (H209) and 0.8 μmol/L (H526)]/topotecan IC_{50} [0.6 μmol/L (H209) and 64 μmol/L (H526)] or camptothecin IC_{25} [10 μmol/L (H209) and 5 μmol/L (H526)]. Vorinostat + topotecan, simultaneous treatment; vorinostat → topotecan or topotecan → vorinostat, sequential treatment with 24 h delay to either drug.

*CI values (mean ± SD from at least three separate experiments done in quadruplicates) computed at 50% of cell kill (CI_{50}) by CalcuSyn software; CI < 0.8 indicated synergism as described in Materials and Methods.

†DRI values (mean ± SD from at least three separate experiments done in quadruplicates) represent the order of magnitude (fold) of dose reduction obtained for IC_{50} (DRI_{50}) in combination setting compared with each drug alone.

To confirm by a different method the synergistic effect observed thus far, a soft-agar clonogenic assay was used to determine the effect of vorinostat and topotecan, alone or in combination, in H526 cells. Vorinostat, when added alone at doses corresponding to 1/10 (250 nmol/L) or 1/5 (500 nmol/L) of the 72 h IC_{50} values (IC_{50} \sim 1/6000) showed in vitro, resulted in \sim 40% and 50% colony formation inhibition, respectively. Topotecan added at a dose of 320 or 640 nmol/L, corresponding to \sim 1/12 or 1/6 of the in vitro IC_{50} values, resulted in 15% and 30% colony formation inhibition, respectively. The addition of both vorinostat and topotecan, combining either the lower or the higher doses of both agents, resulted in 75% or 80% colony formation inhibition, respectively. These data clearly confirmed a statistically significant potentiation of the cytotoxic effect when the two agents were combined (Fig. 3A).

**Cell Cycle Effects Induced by Vorinostat in Combination with Topo-I Inhibitors**

To determine whether the mechanism underlying the observed synergistic interaction might involve cell cycle perturbations, we evaluated the effects of vorinostat and topotecan or camptothecin, either alone or in combination at IC_{50} 72 h concentrations, on the cell cycle kinetics of both H209 and H526 cells (Fig. 2). Vorinostat alone after 48 h induced a slight increase in G_1 phase of the cell cycle in both cell lines in comparison with the controls. On the other hand, topotecan and camptothecin induced a late-middle S-phase cell cycle arrest in both cell lines, whereas an apoptotic effect, measured by the appearance of a hypodiploid population (sub-G_0/G_1 indicated by arrows), was observed only in H209 cells (Fig. 2A). Concomitant treatment with vorinostat did not change the cell cycle perturbations or the apoptotic effect induced by topotecan or camptothecin in H209 cells (Fig. 2A); however, a slight increase in the percentage of cells arrested in S phase and a clear apoptotic effect was observed in the H526 cells treated with a combination of vorinostat and topotecan (Fig. 2B). Similar results were obtained in H526 cells treated with the different combination schedules described earlier (data not shown).

**Vorinostat in Combination with Topotecan Inhibits Soft-Agar Colony Formation and Induces Apoptosis and DNA Damage**

Furthermore, based on the cell cycle analysis data shown in Fig. 2, we next used different approaches to assess the ability of vorinostat and topotecan, individually and in combination, to induce apoptosis in both H526 and H209 cells. Western blot analysis showed increased PARP and caspase-3 cleavage in H526 cells treated simultaneously for 48 h with vorinostat and topotecan at IC_{50} 72 h doses compared with single-drug treatments (Fig. 3B). Notably, the induction of apoptosis by the vorinostat/topotecan combination in H526 cells was also confirmed by the following assays: Annexin V staining, which showed a 2.5-fold increase over the control compared with a slight increase at 72 h.
induced by single drugs (Fig. 3C), DNA fragmentation as seen by ladder formation (Fig. 4B), and destruction of mitochondria (Fig. 4C). In the wild-type p53 H209 cell line, the increased cleavage of PARP was paralleled by an increased expression of p53 induced by combination of vorinostat and topotecan (Fig. 3B).

H2AX is phosphorylated on Ser139 by ATM kinase in response to DNA damage-induced DNA double-strand breaks and is

Figure 2. Effects of vorinostat in combination with topotecan or camptothecin on cell cycle kinetics of H209 and H526 cells. Cell cycle analysis was done after propidium iodide staining on H209 (A) and H526 (B) cells exposed to vorinostat, topotecan, or camptothecin either alone or in combination as indicated. Cells were treated with concentrations of each drug corresponding to IC_{50} for 72 h (vorinostat: 4 μmol/L (H209) and 3 μmol/L (H526); topotecan: 0.007 μmol/L (H209) and 4 μmol/L (H526); and camptothecin: 0.008 μmol/L (H209) and 1.5 μmol/L (H526)) and harvested after 48 h. The relative percentage of cells in different cell cycle phases as well as the percentage of apoptotic events is reported. Arrows, apoptosis, calculated as the hypodiploid population (sub-G_0-G_1) region of the DNA content.
one of the earliest events in the DNA damage signaling and repair (32). An increase of γH2AX in S phase has been shown previously after treatment of cancer cells with topotecan or camptothecin (33). As shown in Fig. 3B, the induction of γH2AX in both H526 and H209 cell lines was observed, only in the combination setting after 48 h, indicating an evident potentiation by vorinostat of topo-I inhibitor-induced DNA damage.

These findings indicate that treatment of SCLC cells with vorinostat/topotecan combination potently induces DNA damage associated with caspase-dependent activation of the apoptotic program.

**Induction of Apoptosis Mediated by the Vorinostat/Topotecan Combination Is Correlated to Enhanced ROS Generation**

Previous studies have suggested that the lethal effect of both HDACI and topo-I inhibitors could be related to oxidative stress as measured by the increase of ROS levels (34–38). Studies were therefore performed to determine whether a similar mechanism might underlie the synergistic cytotoxic and apoptotic effects mediated by vorinostat/topotecan. As shown in Fig. 4A, vorinostat and topotecan (or camptothecin) combination induced a significant increase in ROS levels, compared with single-agent treatments, relative to control untreated H526 and H209 cells. This effect was evident with a time point peak statistically significant of 48 h in H526 cells and of 24 h in H209 cells, probably reflecting different sensitivity toward topo-I inhibitors between the two cell lines. H526 cells treated for 10 h with H2O2 were assumed as positive control (Fig. 4A). Furthermore, coadministration of the antioxidant l-NAC substantially blocked H2O2-mediated as well as the vorinostat/topotecan (or camptothecin)–mediated increase of ROS
In vitro Synergism of Vorinostat plus Topotecan

A

H526

H209

ROS production (% of control)

0 50 100 150 200 250

10 h 24 h 48 h 72 h

B

vorinostat - + - + +
topotecan - - + + +
NAC - - - - +

C

H526 CTR 72h

H526 NAC 72h

H526 vor 72h

H526 topo 72h

H526 vor + topo 72h

H526 vor + topo + NAC 72h

ROS production (% of control)

0 200 400 600 800 1000 1200 1400

1500

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levels in both cell lines. Consistent with these findings, L-NAC also blocked the vorinostat/topotecan-mediated apoptosis in H526 cells as reflected by a reduction of DNA fragmentation shown after 48 h (Fig. 4C) as well as the destruction of mitochondria after 72 h (evaluated as a loss of membrane potential by MitoTracker Red staining and flow cytometric analysis; Fig. 4D), both observed only in combination-treated cells. The latter effect was not observed at early time points (data not shown), indicating that the loss of mitochondrial integrity is a consequence of the apoptotic pathway induced by the vorinostat/topotecan combination.

Collectively, these findings indicate that oxidative injury plays a significant functional role in the enhanced lethality induced by the vorinostat/topotecan combination in SCLC cancer cells.

**Vorinostat Enhanced Topo-I/DNA Cleavable Complex Formation Induced by Camptothecin but Do Not Affect Expression or Catalytic Activity of Topo-I**

To evaluate if vorinostat may affect either expression or activity of topo-I, we first performed Western blotting analysis, showing that treatment with vorinostat (48 h, IC$_{50}$ 2 h) of both H526 and H209 cells does not determine any significant change in topo-I protein level in comparison with control cells (Fig. 5A), in line with what was reported in other cell models (14). We next compared the catalytic activity and the camptothecin sensitivity of topo-I from nuclear extracts of untreated or vorinostat-treated H526 and H209 cells following the electrophoretic mobility of the DNA supercoiled plasmid topo-I substrate. As shown in Fig. 5B, control untreated or 24 h vorinostat-treated cells (IC$_{50}$ 22 h) showed comparable DNA relaxation activities. Similar results were obtained after 48 h of treatment (data not shown). Likewise, increasing concentrations and time titration of vorinostat up to 2 mmol/L do not affect the DNA relaxation monitored adding a purified topo-I protein (Supplementary Fig. S1). Moreover, the catalytic activities of both untreated and vorinostat-treated cells were similarly inhibited by the addition of camptothecin in the reaction mixture (Supplementary Fig. S2). These results indicate that vorinostat-treated cells have the same properties as control untreated cells, with respect to the topo-I catalytic activity and sensitivity to topo-I inhibitors.

To determine whether vorinostat may modulate the amount of topo-I/DNA cleavable complexes induced by topo-I inhibitors, such as camptothecin, we identified topo-I bound to cellular DNA by immunoblot after separating, by cesium chloride gradient centrifugation, cellular extracts of H526 and H209 cells incubated in different conditions (Fig. 5D). The amount of topo-I coincident with the DNA peak, as represented from control untreated H526 and H209 cells in Fig. 5C, is a measure of covalent topo-I/DNA complexes formed. The presence of topo-I/DNA cleavable complexes is detectable also in the control cells in the absence of camptothecin as shown in Fig. 5D (fractions 6-8, control lanes) in line with what was reported in literature (39). Vorinostat alone was unable to modify the amount of cleavable complexes compared with controls. In camptothecin-treated cells, a clear increase in the intensity of the spots corresponding to cleavable topo-I/DNA complexes is observed in H209 cells but not in H526 cells, confirming that they are camptothecin-sensitive and camptothecin-insensitive, respectively. Notably, an increase in topo-I/DNA covalent complexes is observed H526 cells, and slightly in H209 cells, when concomitantly treated with vorinostat and camptothecin relative to cells treated with camptothecin alone (Fig. 5D, fractions 6-8, two last lanes). Free topo-I (unbound) was found to be present in the low molecular weight fraction after cesium chloride centrifugation (Fig. 5D, fractions 15-17).

Collectively, these findings indicate that vorinostat does not affect directly topo-I activity nor changes the content of topo-I but rather potentiate the effect of topo-I inhibitors by increasing, at least in resistant H526 cells, the amount of topo-I/DNA cleavable complexes.

**Discussion**

In the present study, we have shown a synergistic cytotoxicity in SCLC cell lines, regardless of their p53 status, when the HDAC inhibitor vorinostat is combined with the topo-I inhibitor camptothecin or its derivative topotecan. Simultaneous exposures of SCLC cells to equitoxic doses of vorinostat and camptothecin or topotecan as well as low doses of vorinostat and higher doses of any topo-I inhibitors resulted in strong synergistic cytotoxicity. Moreover, the effect of combining vorinostat/topo-I inhibitors is not sequence-dependent, because an evident synergistic effect was achieved when both agents were applied simultaneously or when each was applied 24 h before the other. Notably, we have also shown a strong synergistic cytotoxicity in the H526 cell line that has a marked intrinsic resistance to topo-I inhibitors. The observation that vorinostat might enhance
topo-I inhibitors-induced cytotoxicity even at doses below the IC\textsubscript{50} values suggests that the mechanism of the synergism might depend, at least in part, on the ability of vorinostat to modulate the sensitivity of the cells toward topo-I inhibitors rather than on the concurrent cell killing induced by both agents. Moreover, as shown by previous pharmacokinetic studies (10), the effective doses of vorinostat tested in our \textit{in vitro} study can be easily reached in plasma concentrations.
in patients and with negligible toxicity. In addition, the lack of a schedule dependency in the synergistic interaction between vorinostat and topo-I inhibitors is clinically relevant because a less stringent condition of drug administration would make this combination easily adaptable for clinical applications.

Preclinical studies in multiple cancer cell lines have shown the potentiation by HDACIs of the effects of a wide range of DNA-damaging agents, including topoisomerase inhibitors (31). *In vitro* and *in vivo* preclinical synergistic effects were observed when HDACIs, including vorinostat, were combined with the topo-II inhibitors epirubicin or etoposide (14, 40, 41). A completed phase I clinical study of the HDACIs valproic acid and epirubicin has provided evidence for the safety of this combination (42). Scheduling appeared crucial in these studies and synergy was dependent on the sequence of drug administration (40). Our results are confirmed by the synergism observed in a panel of ovarian cancer cells or pancreatic cancer cells treated simultaneously with a HDACI (even in low doses; refs. 15, 43) and the topo-I inhibitors topotecan or CPT11 (irinotecan hydrochloride hydrate), respectively. On the other hand, in contrast to our data, Kim et al. reported in one glioblastoma cell line that the HDACI trichostatin A given before or simultaneously did not potentiate camptothecin-induced cell killing (41). Furthermore, Bevins and Zimmer showed that HDACIs, added before or simultaneously as camptothecin, limited the number of cells affected by camptothecin, whereas cells treated with a HDACI 24 h after camptothecin were efficiently killed with potentiation of apoptosis (44). The authors suggested that HDACI-induced apoptosis was most effective when HDACI was added to cells already arrested in the G2-M phase of the cell cycle by camptothecin. However, it is worth of mention that several reports clearly show that prolonged treatment of cancer cells with lower, therapeutically achievable camptothecin concentrations causes S-phase arrest, whereas only a brief exposure to high camptothecin concentrations produces a subsequent G2 arrest similar to that shown by Bevins and Zimmer (45–47).

In agreement with these data, we showed a late-middle-S-phase cell cycle arrest induced by topotecan or camptothecin in both H209 and H526 cells. Notably, this effect was either not changed by concomitant treatment with vorinostat or a slight increase of the S-phase cell cycle arrest was observed in resistant H526 cells. Moreover, an apoptotic sub-G1 hypodiploid peak formation was observed in resistant H526 cells treated with a combination of vorinostat/topotecan or vorinostat/camptothecin compared with single drugs. Furthermore, we should point out that the majority of the studies mentioned above did not investigate increasing doses and different cytotoxic ratios of the two agents. Neither did those studies evaluate synergism by the median effect analysis calculating CIs, which is universally accepted as the most reliable and statistically robust approach to investigate the interaction between two or more drugs in a preclinical *in vitro* drug screening test (18). In addition, we showed synergistic interaction between vorinostat and either camptothecin or topotecan by several approaches, including agar colony formation and apoptosis evaluated by various methods. However, we cannot exclude the possibility that the synergistic interaction between these two classes of agents can vary between cell lines or tissue of origin. In this regard, we have observed a less pronounced synergism between vorinostat and topotecan in NSCLC A549 cells compared with that in SCLC cells regardless of the administration schedule of the two drugs (data not shown).

Interestingly, we have shown synergistic interactions between vorinostat and topo-I inhibitors in the context of both wild-type and mutant p53. It is well known that HDAC inhibition helps the stabilization of p53 following DNA damage by preserving the COOH-terminal lysine acetylation (48) and that double-strand breaks induced by topotargeted drugs rapidly trigger p53 protein elevations in cells with wild-type p53 alleles (49). We have indeed observed an increased p53 expression in wild-type p53 H209 cells induced by the concomitant treatment with vorinostat and topotecan. This effect was paralleled by γH2AX induction and increased cleavage of PARP, indicating DNA damage-induced double-strand breaks and apoptosis, respectively. However, a clear induction of γH2AX and cleavage of both PARP and caspase-3 only in the combination setting compared with single-agent treatment was also observed in mutant p53 H526 cells, indicating that the synergistic cytotoxic and apoptotic effects induced by the vorinostat/topotecan combination could occur by either a p53-dependent or a p53-independent mechanism.

Previous studies have shown that the generation of ROS represents an important mechanism by which high concentrations of HDACIs administered alone exert their lethality (34, 36, 37). The mechanism by which this phenomenon occurs is not known with certainty but may involve alterations in the expression of redox-related genes (such as thioredoxin and superoxide dismutase; ref. 34). Moreover, ROS generation on treatment of cancer cells with topo-I inhibitors has been described (35). In our study, ROS generation was strikingly increased in cells simultaneously exposed to vorinostat and topotecan (or camptothecin). Notably, co-administration of the free radical scavenger t-NAC significantly reduced lethality, suggesting a causal role for oxidative damage in the induction of apoptosis. Furthermore, it has been shown that although topo-I-DNA cleavable complexes can be trapped by specific inhibitors such as camptothecin and its derivatives, these transient complexes can also be stabilized by a distinct mechanism related to oxidative DNA lesions and frequent DNA modifications (oxidized bases, mismatches, and strand breaks) and involves cellular events induced by agents that cause ROS generation and subsequent DNA damage (50). Therefore, we speculate that, by combining vorinostat with topo-I inhibitors, we can target topo-I-DNA cleavable complexes by both mechanisms potentiating DNA damage and lethality. Indeed, when cells had been concomitantly treated by vorinostat and camptothecin, an increase in topo-I/DNA covalent complexes was observed relative to cells treated by camptothecin alone. Notably, vorinostat alone did not modulate topo-I expression or activity.
In summary, it is well known that acetylation of core nucleosomal histones on HDACi treatment induces a more relaxed winding of DNA around the histone core, increasing DNA accessibility to cellular enzymes that promote transcription, replication, and repair but eventually also increasing accessibility to DNA-damaging chemotherapeutics such as topo-I inhibitors (11, 31). It has been described that vorinostat as well as other HDACis are able to abrogate the DNA repair process and to inhibit survival mechanisms, thus increasing the cytotoxic effects of DNA-damaging agents and resulting in cell death (11). Together with these potential mechanisms, several lines of evidence indicate that enhanced oxidative damage plays an important functional role in the synergistic interactions observed between vorinostat and topo-I inhibitors in SCLC cells.

In conclusion, to our knowledge, this study is the first to show in a SCLC cell model, and with a novel potential mechanism, a synergistic cytotoxic effect between a HDACi such as vorinostat and a topo-I inhibitor such as topotecan, the latter being the only registered drug for relapsed SCLC. Our results also indicate that the vorinostat/topotecan combination is a promising chemotherapeutic strategy for killing SCLC cells regardless of whether the cells are sensitive or resistant to topo-I inhibitors. Based on our results and the fact that effective treatment options for SCLC patients remain inadequate, a clinical trial of vorinostat and topotecan combination, with a simultaneous schedule of administration, has been started in SCLC.

Disclosure of Potential Conflicts of Interest

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