Combined inhibition of DNA methyltransferase and histone deacetylase restores caspase-8 expression and sensitizes SCLC cells to TRAIL

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising drug for the treatment of tumors; however, a number of cancer cells are resistant to this cytokine. Among the mechanisms of resistance of small cell lung carcinomas (SCLCs) to TRAIL is the lack of caspase-8 expression. Although methylation of the caspase-8 promoter has been suggested as the main mechanism of caspase-8 silencing, we showed that reduction of the enzymes involved in DNA methylation, DNA methyltransferases (DNMT) 1, 3a and 3b, was not sufficient to significantly restore caspase-8 expression in SCLC cells, signifying that other mechanisms are involved in caspase-8 silencing. We found that combination of the DNMT inhibitor decitabine with an inhibitor of histone deacetylase (HDAC) significantly increased caspase-8 expression in SCLC cells at the RNA and protein levels. Among all studied HDAC inhibitors, valproic acid (VPA) and CI-994 showed prolonged effects on histone acetylation, while combination with decitabine produced the most prominent effects on caspase-8 re-expression. Moreover, a significant reduction of survivin and cIAP-1 proteins level was observed after treatment with VPA. The combination of two drugs sensitized SCLC cells to TRAIL-induced apoptosis, involving mitochondrial apoptotic pathway and was accompanied by Bid cleavage, activation of Bax, and release of cytochrome c. Both initiator caspase-8 and -9 were required for the sensitization of SCLC cells to TRAIL. Thus, efficient restoration of caspase-8 expression in SCLC cells is achieved when a combination of DNMT and HDAC inhibitors is used, suggesting a combination of decitabine and VPA or CI-994 as a potential treatment for sensitization of SCLC cells lacking caspase-8 to TRAIL.

Introduction

Lung cancer is one of the leading causes of cancer death in the Western world. Based on histopathology, it is divided into non-small cell lung carcinoma and small cell lung carcinomas (SCLCs), where SCLCs account for ~15% of lung cancer cases. The most widely used regimen for the treatment of SCLCs is radio- and chemotherapy, including etoposide and cisplatin. Compared with other cancers, SCLCs initially respond well to treatment; however, long-term survivors have considerable mortality due to late relapses and secondary malignancies. The overall 5 years survival of patients with SCLC is 5–10% (1,2). Therefore, searching for alternative drugs to treat this disease is of great importance.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been described as a promising cytokine, which specifically kills tumor cells while sparing most normal cells (3). Upon TRAIL binding to its surface death receptors (DRs), TRAIL receptor-1 (DR4) or TRAIL receptor-2 (DR5), adapter protein Fas-associated death domain (FADD) and caspase-8 are recruited, forming a death-inducing signaling complex (DISC) (4). The formation of this DISC complex leads to caspase-8 activation and initiation of the apoptotic pathway. Depending on the level of active caspase-8, direct (type I cells) or indirect (via mitochondria, type II cells) activation of the effector caspase-3 may follow. In the mitochondria-dependent pathway, active caspase-8 cleaves Bid, which induces activation of Bax and permeabilization of mitochondria, followed by activation of caspase-9 and caspase-3 processing (5). Although TRAIL specifically kills cancer cells, some tumor types are resistant to this cytokine. One explanation for the resistance of cancer cells to TRAIL treatment is a lack of caspase-8 expression. Most SCLC cell lines and tumor samples are deficient in caspase-8 expression due to methylation of CpG islands in the caspase-8 gene (6,7). Our previous studies have shown that even caspase-8 expressing SCLC cells could be resistant to TRAIL (8). It has been found that DNA-damaging drugs doxorubicin and etoposide are able to sensitize these cells to TRAIL-mediated cell death, by increasing DR5 levels and downregulating cFLIP. However, these conventional chemotherapeutic drugs were not capable of restoring TRAIL sensitivity in SCLC cells lacking caspase-8. Therefore, the relevance of TRAIL therapy in SCLCs lacking caspase-8 needs to be elucidated.

Previously, it has been shown that either an inhibitor of DNA methyltransferases (DNMT), decitabine or interferon (IFN) γ is able to induce caspase-8 expression and restore the sensitivity of neuroblastomas to TRAIL (9,10). Another study demonstrated that coadministration of an histone deacetylase (HDAC) inhibitor with IFNγ significantly increased the sensitivity of medulloblastomas to TRAIL (11). In the present study, we show that inhibition of the main enzymes involved in DNA methylation, namely DNMT1, 3a and 3b, is not sufficient to restore caspase-8 expression (or TRAIL sensitivity) in a panel of SCLC cell lines, suggesting that some other mechanisms might be involved in caspase-8 silencing. We found that a combination of the DNMT inhibitor decitabine with an HDAC inhibitor, either valproic acid (VPA) or CI-994, efficiently restored expression of caspase-8 in SCLC cell lines and reduced the levels of cIAP-1 and survivin. Moreover, the combination of these two epigenetic drugs sensitized SCLC cells to TRAIL. We also demonstrate the involvement of the mitochondrial death pathway and the important role of both initiator caspases-8 and -9 in TRAIL-induced cell death. A combination of an inhibitor of DNA methylation, decitabine, with an HDAC inhibitor, VPA or CI-994, is proposed as a potential treatment option for sensitization of SCLC cells lacking caspase-8 to TRAIL.

Materials and methods

Cell culture and treatments

Human SCLC cell lines H69 (ECACC), H82 (ATCC), H1417 (ATCC), H2171 (ATCC) and U1906 (UU collection) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) (all obtained from Gibco, Karlsruhe, Germany) at 37°C, 5% CO₂ and 95% humidity. Cells were treated with decitabine, VPA, trichostatin A (TSA) (all from Sigma-Aldrich, St Louis, MO), vorinostat, and CI-994 (LC Laboratories, Woburn, MA), human recombinant Killer TRAIL (provided by Dr L. Andera), caspase-8 inhibitor z-IETD-fmk and caspase-9 inhibitor z-LEHD-fmk (both obtained from Enzyme Systems Products, Livermore, CA) or vehicle (dimethyl sulfoxide) for the time points and concentrations indicated in the figure legends.

Surface TRAIL receptor expression

The level of expression of surface TRAIL receptors was detected by flow cytometry as described previously (8). Briefly, after washing with phosphate-buffered saline (PBS), cells were incubated (40 min, 4°C) with primary
Sensitization of caspase-8-lacking SCLC cells to TRAIL

Fig. 1. SCLC cells lack caspase-8 and display differential expression of TRAIL signaling molecules. (A) The level of procaspase-8 and procaspase-10 protein expression in H69, H82, H1417, H2171 and U1906 cell lines detected by western blot. (B) Reverse transcription–polymerase chain reaction analysis of caspase-8 mRNA expression in SCLC cell lines. Jurkat cells were used as a positive control for expression of caspase-8 mRNA and protein levels. (C) The level of FLIPs, FADD, DR4, DR5, Bcl-2, Bcl-xL, and Mcl-1 proteins in studied cell lines. Equal loading was verified using anti-β-actin antibody. Results shown are the mean ± standard error of the mean of three independent experiments.

Annexin V/PI staining
Annexin V/Propidium iodide double staining was carried out using an Annexin-V-FLUOS Staining Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. Cells were analyzed by flow cytometry (FACScan; Becton Dickinson), and data were evaluated using Cell Quest software.

Analysis of mitochondrial membrane potential
For analysis of mitochondrial membrane potential, cells were stained for 20 min with 25 nM TMRE (Molecular Probes) in PBS at 37°C, 5% CO2. After washing twice with PBS, samples were analyzed by flow cytometry.

Caspase activity assay
Cells were washed with ice-cold PBS, resuspended in 25 μl PBS and, after lysis by freezing in liquid nitrogen, were loaded onto a microtiter plate. The caspase-3 substrate DEVD-AMC (50 μM; Peptide Institute, Osaka, Japan) was added and fluorescence detected using a Fluoroscan II plate reader (Labsystems, Waltham, MA) with 355 nm excitation and 460 nm emission wavelengths. Fluorescent units were converted to picomoles of released AMC and subsequently related to the amount of protein in each sample. Finally, caspase activity was expressed as the fold-increase relative to appropriate controls.

RNA interference
Cells were seeded onto six-well plates in growth media without antibiotics. Nontargeting siRNA, anti-caspase-8, anti-caspase-9 siRNA (all from Dharmacon RNA Technologies, Lafayette, CO) or anti-HDAC2 (Sigma-Aldrich) were diluted in 100 μl of OPTI-MEM (Gibco) and mixed with 5 μl of INTERFERin siRNA Transfection Reagent (Polyplus Transfection, Illkirch, France). After 10 min of incubation, the complexes were added to the cells. The final concentration of siRNA in the medium was 50 nM. Twenty-four hours after transfection, the medium was replaced and the desired treatments were administered.

Immunoblotting
Cells were lysed in Complete lysis buffer (Roche Diagnostics) with protease inhibitor cocktail (Complete-M; Roche Diagnostics). The protein concentration was determined in each sample (BCA protein assay; Pierce Biotechnology, Rockford, IL). Samples were then mixed with Laemmli buffer and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blotting. For immunodetection, the following antibodies were used: anti-cleaved PARP, anti-cleaved caspase-3, anti-Bid (all obtained from Cell Signaling Technology, Danvers, MA), anti-DR4, anti-DR5, anti-Mcl-1 (Sigma–Aldrich), anti-surivinv (Abcam, Cambridge, MA), anti-CLAP1 (BD Pharmingen, San Jose, CA), anti-caspase-10 (MBL, Naka-Ku Nagoya, Japan), anti-FLICE-like inhibitory protein (FLIP; Alexis Biochemicals, San Diego, CA), anti-FADD, anti-cytokine c (all from BD Pharmingen), anti-caspase-8 (provided by Prof P Krammer), anti-Bcl2, anti-Bcl-XL (all from Santa Cruz Biotechnology, Santa Cruz, CA), anti-DNMT1, anti-DNMT3a and anti-DNMT3b (all from Novus Biologicals, Littleton, CO). Recognized proteins were detected using horseradish peroxidase-labeled anti-mouse or anti-rabbit secondary antibodies (Pierce Biotechnology) and an enhanced chemiluminescence kit (western blot detection reagent; GE Healthcare, Piscataway, NJ). Equal loading was verified using anti-β-actin (Sigma–Aldrich) antibodies. For detection of cytotoxic and mitochondrial cytochrome c, cells were washed twice with PBS, incubated (5 min, 4°C) in a buffer (250 mM sucrose, 70 mM KCl, 100 μg/ml digitonin in PBS), centrifuged (5 min, 7000g) and the supernatant was collected (cytosolic fraction). Mitochondrial fractions were prepared by lysis of the pellet in Complete Lysis-M buffer with protease inhibitor cocktail (Roche Diagnostics).

Real-time quantitative polymerase chain reaction
Reversed transcribed complementary DNA from the samples were used as templates. Caspase-8 (C8-1, 5’-cagctaggccagggagtt-3’ and C8-2, 5’-gattctacca gcttgccat-3’) and 18S ribosomal RNA (18S-1, 5’-ccctggcggcttt-3’ and 18S-2, 5’-actgtactatgcgtctcc-3’) primers (Invitrogen, Carlsbad, CA) were designed to match the target complementary DNA sequence. About 20 ng of the reversed transcribed complementary DNA template was mixed with SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) and amplified using a 7500 Real-Time PCR System (Applied Biosystems) with the following program: 40 cycles, with each cycle consisting of a denaturation step at 95°C for 15 s and an annealing/extension step at 60°C for 1 min. Messenger RNA (mRNA) expression levels of each gene in treated cells are presented as the fold-increase relative to untreated cells, after normalization against 18S RNA.

Immunofluorescence microscopy
Cells were seeded on coverslips and, after desired treatments, were washed with PBS and fixed with 4% paraformaldehyde (15 min, room temperature). After washing twice, cells were stained using anti-Bax (BD Pharmingen) and anti-cleaved caspase-3 (Cell Signaling Technology) antibodies, followed by detection with AlexaFluor594-conjugated goat anti-rabbit IgG or AlexaFluor488-conjugated donkey anti-mouse IgG secondary antibodies (all from Molecular Probes). Nuclei were counterstained with Hoechst 33342 (10 μg/ml in PBS) for 5 min incubation at room temperature. After washing with PBS, slides were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and examined under a Zeiss LSM 510 META confocal laser scanner microscope (Carl Zeiss, Jena, Germany).

Statistical evaluation
The results of three independent experiments were expressed as the mean ± standard error of the mean. Statistical evaluation was performed using a non-paired t-test.
Our previous studies showed that DNA-damaging drugs, etoposide or doxorubicin, induce apoptosis synergistically with TRAIL in SCLC cells-expressing caspase-8 (8); however, these synergistic effects failed in cells lacking caspase-8. Therefore, the aim of the current study was to evaluate the rationality of using TRAIL therapy for the treatment of SCLC cells lacking caspase-8.

A panel of five SCLC cell lines (H69, H82, H1417, H2171 and U1906) was examined for caspase-8 and caspase-10 expression in order to verify their absence at the protein level. We confirmed that all studied cell lines either expressed no (H69, H82, U1906) or negligible (H1417, H2171) levels of caspase-8 mRNA (quantitative polymerase chain reaction, after 40 cycles) and protein (western blotting) compared with Jurkat cells, which were used as a positive control (Figure 1A and B). All results are representative of three independent experiments.

**Results**

**Characterization of SCLC cell lines lacking caspase-8: expression of molecules involved in TRAIL signaling**

Our previous studies showed that DNA-damaging drugs, etoposide or doxorubicin, induce apoptosis synergistically with TRAIL in SCLC cells-expressing caspase-8 (8); however, these synergistic effects failed in cells lacking caspase-8. Therefore, the aim of the current study was to evaluate the rationality of using TRAIL therapy for the treatment of SCLC cells lacking caspase-8.

A panel of five SCLC cell lines (H69, H82, H1417, H2171 and U1906) was examined for caspase-8 and caspase-10 expression in order
Histone H3 in U1906 cells treated for 48 h with indicated HDAC inhibitors and VPA (1 mM). (used: vorinostat (SAHA, 1 M), CI-994 (2 M), trichostatin A (TSA, 50 nM) and VPA (1 mM)). The following HDAC inhibitors were used: vorinostat (SAHA, 1 M), CI-994 (2 M), trichostatin A (TSA, 50 nM) and VPA (1 mM). (C) The level of caspase-8 expression and acetylation of histone H3 in U1906 cells treated for 48 h with indicated HDAC inhibitors and/or decitabine. For (A–C), equal loading was verified using anti-β-actin antibody. All data are representative of three independent experiments.

Fig. 3. Effect of DNA-modifying agents on histone acetylation and the expression of DNMT. (A) The level of DNMT 1, 3a and 3b and acetylation of histone H3 in cells treated with decitabine (DCB, 0.5 μM) and/or VPA (1 mM). (B) Analysis of histone H3 acetylation in U1906 cells treated for 1, 6, 24 and 48 h with HDAC inhibitors. The following HDAC inhibitors were used: vorinostat (SAHA, 1 μM), CI-994 (2 μM), trichostatin A (TSA, 50 nM) and VPA (1 mM). (C) The level of caspase-8 expression and acetylation of histone H3 in U1906 cells treated for 48 h with indicated HDAC inhibitors and/or decitabine. For (A–C), equal loading was verified using anti-β-actin antibody. All data are representative of three independent experiments.

A combination of decitabine and VPA was effective at restoring caspase-8 expression in SCLC cell lines, while only a minimal effect was observed after decitabine alone.

Because frequent inactivation of the CASP8 gene by methylation of its promoter was reported in neuroendocrine tumors of the lung (12,13), the demethylation agent decitabine (5-aza-2'-deoxycytidine) was examined for its ability to restore caspase-8 protein expression, using two cell lines with undetectable levels of caspase-8 mRNA (U1906 and H82) as well as H2171 cells, which express negligible amounts of caspase-8 mRNA. In order to test for restoration of caspase-8 expression, the selected SCLC cell lines (U1906, H82, H2171) were incubated (48 h) with decitabine (DCB, 0.5 μM) and caspase-8 expression was detected by western blotting. While no significant effect was apparent in H82 cells, decitabine treatment slightly increased the expression of pro-caspase-8 protein in U1906 or H2171 cells (Figure 2A and supplementary Figure 1A) is available at Carcinogenesis Online. However, the amount of caspase-8 induced by a pharmacologically relevant dosage of decitabine was insufficient to sensitize U1906 cells to the proapoptotic action of TRAIL (supplementary Figure 1B is available at Carcinogenesis Online). Taking into account previous reports, which showed that other epigenetic modifications of DNA might be involved in gene silencing, we further explored whether inhibition of HDAC activity, which is known to result in accumulation of histone acetylation and a permissive gene expression state (14), could help restore caspase-8 expression in SCLCs. VPA is an approved mood-stabilizing drug with HDAC inhibitory activity, which has been recently tested as an antineoplastic agent and used in phase 1/2 clinical trials in combination with decitabine, in patients with advanced leukemia (15). To further investigate the effects of a combination of decitabine and VPA on caspase-8 expression and cell sensitization to TRAIL, U1906 and H2171 cells, which express both DR4 and DR5 at their cell surfaces were chosen (supplementary Figure 2 is available at Carcinogenesis Online). Indeed, we showed that simultaneous treatment (48 h) of U1906 and H2171 cells with decitabine (DCB, 0.5 μM) and VPA (1 mM) significantly restored the expression of caspase-8 mRNA and protein compared with treatment with either of the agents alone (Figure 2A, B). Together, these results show that VPA and decitabine co-operate to reactivate caspase-8 expression at the transcriptional and translational levels. The level of Mcl-1 protein was also significantly upregulated upon treatment of U1906 cells with decitabine and VPA; however, it was not changed in H2171 cells expressing relatively high basal levels of Mcl-1 (supplementary Figure 3 is available at Carcinogenesis Online). At the same time, we did not find any significant changes in the expression levels of the other components of TRAIL signaling pathway, such as DR4, DR5 (total and surface), cFLIP(L, cFLIPS, Bcl-xL or caspase-10, after combined treatment with decitabine and VPA (Figure 2C and D).

Decitabine- and VPA-induced restoration of caspase-8 is associated with substantial histone H3 acetylation and downregulation of DNMT

To clarify the molecular mechanisms underlying the co-operative effects of VPA and decitabine at restoring caspase-8 expression, histone acetylation and the expression of DNMT upon treatment with either agent alone or in combination were analyzed. Treatment with VPA alone (1 mM, 48 h) induced significant acetylation of histone 3 in U1906 and H2171 cells (Figure 3A), whereas treatment with decitabine alone significantly downregulated expression of DNMT 1, 3a and 3b in both studied cell lines (Figure 3A). Notably, combined treatment with VPA and decitabine did not additionally affect histone acetylation, or the expression of DNMT, compared with each agent used alone. Thus, both significant inhibition of HDACs (as measured by acetylation of histone 3) and downregulation of DNMTs were observed in cells treated with a combination of VPA and decitabine. Further analysis using several different HDAC inhibitors revealed that effect of VPA (1 mM) and CI-994 (2 μM) was associated with the most stable and prolonged histone acetylation, which was maintained up to 48 h, whereas SAHA (1 μM) and TSA (50 nM) promoted substantial but temporary effects at early treatment times (6 and 24 h), which were considerably reduced by 48 h (Figure 3B). Importantly, all HDAC inhibitors used, only application of CI-994 in combination with decitabine restored caspase-8 expression to a level similar to that induced by VPA (Figure 3C). Overall, these results indicate that combined treatment with VPA and decitabine promotes strong and sustained histone acetylation, which, in addition to inhibition of DNMT, induces a significant increase in caspase-8 expression.

The combination of decitabine and VPA restored the sensitivity of SCLC cells to TRAIL-induced apoptosis

To address changes in the sensitivity of cells with re-expressed caspase-8 to the apoptotic effects of TRAIL, U1906 and H2171 cells were pretreated (48 h) with a combination of decitabine (DCB, 0.5 μM) and VPA (1 mM) and then treated (24 h) with TRAIL (200 ng/ml). Following this triple combination treatment, considerably
enhanced TRAIL-induced apoptosis was observed in both U1906 and H2171 cells compared with single or double treatments, as demonstrated by a substantial increase in caspase-8, -9 and -3 processing as well as caspase-3 activity and PARP cleavage (Figure 4A and B). These effects were paralleled by a significant increase in the amount of Annexin V-positive apoptotic cells (Figure 4C). We showed that following treatment with VPA alone or its combination with decitabine and TRAIL, the levels of cIAP-1 and survivin were significantly increased in both cell lines. Figure 4A shows the levels of cIAP-1 and survivin protein expression in U1906 cells treated with decitabine (DCB, 0.5 μM), VPA (1 mM) or their combination followed by treatment with TRAIL (24 h, 200 ng/ml). Equal loading was verified using anti-GAPDH-antibody.

Fig. 4. The combination of decitabine and VPA restores SCLC cell sensitivity to TRAIL. The U1906 and H2171 were pretreated (48 h) with decitabine (DCB, 0.5 μM), VPA (1 mM) or their combination and treated with TRAIL (24 h, 200 ng/ml). (A) Cleavage of PARP and processing of caspase-8, -3 and -9 in U1906 and H2171 cells. Anti-β-actin antibodies were used to verify equal loading. (B) Caspase-3-like activity (fold increase with respect to control) in U1906 and H2171 cells after treatment with either agent alone or in combination (for details see Materials and Methods). (C) Apoptotic cell death assessed by Annexin V (x-axis) and propidium iodide (y-axis) staining. U1906 cells were treated (48 h) with decitabine (DCB, 0.5 μM), VPA (1 mM) or their combination, followed by treatment with TRAIL (24 h, 200 ng/ml). Results shown are the mean ± standard error of the mean of three independent experiments. (D) The level of cIAP-1 and survivin protein expression in U1906 cells treated with decitabine (DCB, 0.5 μM), VPA (1 mM) or their combination followed by treatment with TRAIL (24 h, 200 ng/ml). Equal loading was verified using anti-GAPDH-antibody.
Decitabine- and VPA-mediated enhancement of TRAIL-induced apoptosis is associated with activation of mitochondrial apoptotic pathway

Containing with antibodies to active Bax and active caspase-3 showed that after pretreatment with decitabine and VPA (48 h), incubation with TRAIL induced substantial activation of these proteins (Figure 6A). Note that apoptosis-related morphological changes were already observed 3 h after TRAIL administration. Further studies showed that pretreatment of U1906 cells with decitabine and VPA resulted in enhanced cleavage of Bid and release of cytochrome c after TRAIL treatment (Figure 6B). Additionally, a rapid drop in mitochondrial membrane potential was detected in cells with restored caspase-8 after induction of apoptosis with TRAIL (Figure 6C). Altogether, these data, coupled with previously obtained results regarding caspase-9 silencing, demonstrate that decitabine- and VPA-induced sensitization of SCLC cells to TRAIL-mediated apoptosis engages the mitochondrial pathway and depends on mitochondrial outer membrane permeabilization.

Discussion

Although SCLCs initially respond well to conventional chemo- and radiotherapy, relapses occur in most cases, accompanied by severe resistance during the course of the disease. Therefore, alternative strategies are required for the treatment of these tumors. Because DNA is a primary target of the therapeutic drugs that are used for the treatment of lung carcinomas, an alternative strategy for killing lung cancer cells may be targeting surface DRs. Despite the fact that TRAIL has been described as a potential drug to preferentially induce apoptosis in cancer cells, while sparing normal cells (3), accumulating evidence demonstrates that many cancers are resistant to treatment with this cytokine (16). Our previous studies have shown that conventional DNA-damaging drugs did not restore the TRAIL sensitivity of SCLC cells lacking caspase-8 (8).

TRAiL is a cytokine that is expressed in normal human tissues, including peripheral blood leukocytes, spleen, lung, prostate (17) and has also been found in tumor tissues (18). TRAIL has been shown previously to be involved in the enhanced cytotoxicity of IFN-beta-stimulated human dendritic cells to tumor cells (19). However, TRAIL has also been linked to the survival and proliferation of SCLC cells lacking caspase-8, which is mediated by ERK and dependent on DR5 expression (20). Thus, it was suggested that, in cancer cells, this cytokine can either trigger apoptosis or, in cells lacking caspase-8, promote tumor growth. Therefore, finding potential drugs that may restore expression of caspase-8 in SCLCs and sensitize these cells to TRAIL is of great importance. Because treatments using ‘epigenetics’ drugs are now in clinical trials for the therapy of various tumors, we aimed to establish the importance. Because treatments using ‘epigenetics’ drugs are now in clinical trials for the therapy of various tumors, we aimed to establish the importance.
restoring caspase-8 expression. The main mechanism of action of decitabine is incorporation into DNA and inhibition of DNMT causing hypomethylation of DNA; however, it should be noted that this drug also might be incorporated into RNA during transcription. A HDAC inhibitor VPA is used in clinics as an agonist of GABA receptor; however, in contrast to VPA, another agonist of GABA receptor, gamma-aminobutyric acid alone or in combination with decitabine was not able to restore the expression of caspase-8 in SCLC cells (data not shown). Moreover, structurally different inhibitor of HDAC, CI-994, acted synergistically with decitabine in inducing caspase-8 (see Figure 3C), suggesting that HDAC inhibition plays a critical role in this effect.

Previous studies demonstrated that DNA methylation and histone deacetylation appear to act as synergistic layers during gene silencing in cancer (21,22). Recently, the combination of HDAC inhibitors with inhibitors of DNMTs was suggested for use in clinical trials. For example, the combination of VPA and decitabine is now in phase I and II trials for the treatment of patients with leukemia or advanced cancers (15,23). In addition, the combination of CI-994 with gemcitabine is in phase III clinical trials for the treatment of patients with...
advanced non-small cell lung carcinomas (http://clinicaltrials.gov/ ct2/show/NCT00005093). Altogether, these data demonstrate that treatment with a combination of these two epigenetic drugs might have potential for restoring caspase-8 expression in SCLCs.

Although three subclones of mammalian HDAC have been described (24) in our experiments, the highest level of caspase-8 expression restoration was observed when class I selective HDAC inhibitors, VPA or CI-994, were used (25). In addition to inhibiting the catalytic activity of HDACs, treatment with VPA at therapeutically relevant concentrations has been shown to induce specific degradation of HDAC2 (26). Furthermore, the non-catalytic N-terminus of the chief enzyme that maintains mammalian DNA methylation, DNMT1, binds to HDAC2 and establishes a repressive transcription complex (27). Based on these findings, we hypothesized that, in addition to methylation of the caspase-8 gene, HDAC2 activity may also play a role in caspase-8 silencing. However, results obtained here demonstrate that silencing of HDAC2, in combination with decitabine, does not significantly increase caspase-8 levels (supplementary Figure 5 is available at Carcinogenesis Online), indicating that several HDACs may be simultaneously involved in silencing of this protease. Considering that a potent HDAC inhibitor, vorinostat (SAHA) and TSA had less prominent effects on restoring caspase-8, we investigated differences in the action of all of these inhibitors. Previously, it was reported that transient hyperacetylation of histones H3 and H4 was detectable upon TSA treatment (28). In agreement with these observations, we found quite rapid (within 1 h) histone acetylation after treatment of cells with TSA and vorinostat; however, with time, this effect was significantly reduced. In contrast, the other two HDAC inhibitors, VPA and CI-994, had a prominent effect on histone acetylation even after 48 h of treatment. Moreover, both VPA and CI-994 had more pronounced effects on the restoration of caspase-8. Based on these observations, we conclude that stable histone acetylation is required for efficient restoration of caspase-8 in SCLCs.

Our findings demonstrate that sensitization of SCLC cells to TRAIL-mediated apoptosis requires caspase-8 and -9 and involves a mitochondria-mediated apoptotic pathway. It was shown that HDAC inhibitors may decrease the expression of antiapoptotic Bcl-2 and Bcl-xL proteins or increase proapoptotic Bcl-2 family members and, therefore, enhance the intrinsic apoptotic pathway (29–31). Interestingly, HDAC inhibitors were also shown to induce selective apoptosis in tumors through activation of the DR pathway (32,33). Although in our studies, we did not observe downregulation of antiapoptotic Bcl-2 family proteins, combined treatment with two DNA-modifying drugs followed by TRAIL treatment led to Bax activation, Bid processing and release of cytochrome c, indicating that TRAIL-mediated apoptosis also engages the mitochondrial pathway. In addition to the enhancement of apoptosis at the level of mitochondria, other DISC components have been shown to play a role in sensitization to TRAIL. Although in our studies, using a panel of SCLC cell lines, the level of DR4 and DR5 surface receptors and cFLIP was not changed, findings from other groups demonstrated that HDAC inhibitors might sensitize cells to TRAIL-mediated apoptosis by increasing the expression of DR4 or DR5 and downregulating cFLIP (34–38), indicating that the effect of the DNA-modifying drugs tested on DISC components is also cell type-dependent. Furthermore, we found that treatment with VPA reduced the levels of antiapoptotic proteins survivin and cIAP-1, indicating that along with induced expression of caspase-8, inhibition of these two IAP family members might contribute to sensitization of cells to TRAIL-mediated apoptosis. Moreover, it is probably that Mcl-1, another antiapoptotic member of Bcl-2 family, does not play a critical role in sensitivity of SCLC cells to TRAIL treatment. Similar observation has recently been described (39). The authors showed that sensitization of glioma cells to TRAIL by treatment with bortezomib accompanied by a robust increase in the level of Mcl-1.

In summary, our study shows that a combination of HDAC inhibitor VPA or CI-994 with DNMT inhibitor decitabine efficiently restores expression of caspase-8 in SCLC cell lines. The combination of these two epigenetic drugs is suggested as a new useful strategy for sensitization of SCLC cells to TRAIL-mediated apoptotic cell death.

Supplementary material
Supplementary Figures 1–5 can be found at http://carcin.oxfordjournals.org/

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