Valproic Acid, an Antiepileptic Drug with Histone Deacetylase Inhibitory Activity, Potentiates the Cytotoxic Effect of Apo2L/TRAIL on Cultured Thoracic Cancer Cells through Mitochondria-Dependent Caspase Activation

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Abstract
Inhibitors of histone deacetylases have been shown to enhance the sensitivity of cancer cells to tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)-mediated cytotoxicity. Valproic acid (VA), a commonly used antiepileptic agent whose pharmacokinetics and toxicity profiles are well described, is a histone deacetylase inhibitor. This project aims to evaluate if VA can potentiate Apo2L/TRAIL–mediated cytotoxicity in cultured thoracic cancer cells and to elucidate the underlying molecular mechanism responsible for this effect. VA sensitized cultured thoracic cancer cells to Apo2L/TRAIL, as indicated by a 4-fold to a >20-fold reduction of Apo2L/TRAIL IC_{50} values in combination-treated cells. Although VA (0.5–5 mM) or Apo2L/TRAIL (20 ng/ml) induced less than 20% cell death, VA + Apo2L/TRAIL combinations caused 60% to 90% apoptosis of cancer cells. Moreover, substantial activation of caspases 8, 9, and 3, which was observed only in cells treated with the drug combinations, was completely suppressed by Bcl2 overexpression or by the caspase 9 inhibitor. Both the caspase 9 inhibitor and Bcl2 completely abrogated the substantial cytotoxicity and apoptosis induced by this combination, thus highlighting the pivotal role of the type II pathway in this process. These findings provide a rationale for the development of VA and Apo2L/TRAIL combination as a novel molecular therapeutic regimen for thoracic cancers.

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Keywords: Histone deacetylase inhibitor, Apo2L/TRAIL, lung cancer, esophageal cancer, malignant pleural mesothelioma, mitochondria, Bcl2, caspases.

Introduction
Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is expressed by natural killer cells, T cells, neutrophils and monocytes in responses to interferons and play physiologic roles of tumor surveillance and immune regulation [1,2]. The extracellular domain of membrane-bound TRAIL can be proteolytically cleaved from the cell surface to generate soluble TRAIL. The soluble TRAIL extracellular domains homotrimerize through an internal zinc atom bound to the cysteine residue at position 230 of each subunit, and this process is crucial for trimer stability and biologic activity [3,4]. The receptor repertoire of TRAIL is complex and consists of five members: two functional death receptors (DR4, DR5) that contain a conserved intracellular death domain motif; two decoy receptors (DcR1, DcR2) that can bind TRAIL but lack the functional intracellular death domain and are thus incapable of transducing a death signal; and the fifth is a soluble protein [osteoprotegerin (OPG)] that binds TRAIL but at a very low affinity at physiologic condition [4]. Each homotrimerized TRAIL molecule engages three receptors, each at the interface of its two subunits. The molecular events downstream of DR4/DR5 activation by TRAIL are well described [4–6]. Binding of homotrimerized TRAIL to its appropriate receptors DR4 and/or DR5 results in receptor aggregation, recruitment of FADD, FLIP, and procaspases 8/10 to form the death-inducing signaling complex (DISC) leading to caspase 8/10 activation (by autocalytic cleavage, the induced proximity hypothesis [7], or by homodimerization of the procaspases, the unified model [8]) [9]. The DISC-activated caspase 8 then activates caspases 3, 6, and 7 by proteolytic cleavages of respective procaspases leading to induction of apoptosis through a signaling cascade known as the extrinsic pathway, in contrast to the mitochondria-mediated.

Abbreviations: TRAIL, TNF-related apoptosis inducing ligand; NSCLC, non–small cell lung cancer; EsC, esophageal cancer; MPM, malignant pleural mesothelioma; HDACI, histone deacetylase inhibitor; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; MTT, (4,5-dimethylthiazo-2-yl)-2,5-diphenyl tetrazolium bromide; VA, valproic acid; SMAC/DIABLO, second mitochondria-derived activator; GFP, green fluorescence protein

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intrinsic death signaling pathway activated following cytotoxic stresses [10,11]. Moreover, proteolytically active caspase 8 can also process Bcl2-interacting domain (BID) to yield truncated BID (tBID), which translocates to the mitochondria and, through interaction with Bak and Bax [12–14] on the mitochondria outer membrane, mediates the release of multiple proapoptotic proteins including cytochrome c, apoptosis-inducing factor, and second mitochondria-derived activator (SMAC/DIABLO), thus linking the extrinsic to the intrinsic death signaling pathways [10,11,15,16]. Cytochrome c, together with cytosolic apoptotic protease activating factor 1, procaspase 9, and ATP, forms the apoptosome that activates caspase 9. Activated caspase 9 processes caspases 3/7, which proteolytically cleave and activate caspase 8, thus forming a positive amplification feedback loop to further activate the apical caspase 8 [1]. Death ligand–mediated induction of apoptosis is further classified into type I or type II depending on the involvement of the intrinsic (mitochondria mediated) death signaling cascade in the effective execution of apoptosis. Type I cells undergo death receptor–mediated apoptosis independent of mitochondria (and thus not sensitive to Bcl2/BclXL), whereas type II cells rely on the intrinsic pathway for efficient apoptosis (and apoptosis is abrogated by Bcl2, BclXL or by the selective caspase 9 inhibitor Z-LEHD-fmk) [17].

Recombinant human TRAIL, in its Zn^{2+}-containing homotrimer form known as Apo2L/TRAIL (Genentech, Inc., South San Francisco, CA), in contrast to other forms of recombinant TRAIL, has been shown to be tumor selective in that it rapidly and profoundly induces apoptosis of susceptible cancer cells while sparing normal cells especially primary human hepatocytes [3,18,19]. Systemic administrations in nonhuman primates were well tolerated with no apparent toxicity to the liver or the bone marrow [19,20]. Malignant tumors of diverse histology (cultured cell lines and tumor specimen) amply express DR4 and/or DR5, yet significant proportions of receptor-positive cultured cancer cells exhibit resistance to the cytotoxic effect of Apo2L/TRAIL in vivo [21]. The molecular basis of this intrinsic or acquired resistance to TRAIL-induced cytotoxicity in various cancer cell lines is complex and multifactorial [22]. Fortunately, this limitation can be overcome by combining recombinant TRAIL receptor ligand with cancer chemotherapeutic agents (standard cytotoxic drugs like cisplatin [23–26], CTP-11 [19], or others [24,26–29] as well as experimental anticancer drugs [30]) [31,32]. Whereas the underlying mechanisms responsible for the synergistic interactions between chemotherapeutics and TRAIL receptor agonists to mediate profound induction of apoptosis are incompletely understood, it appears that recruitment/activation of the intrinsic death pathway (mitochondria mediated) in combination-treated cells plays the crucial role [28,33,34].

Histone deacetylase inhibitors (HDACIs) are structurally diverse chemical compounds that share common biologic properties of inducing core histone hyperacetylation leading to gene expression and of mediating potent antitumor effects [35–37]. Some HDACIs are either naturally occurring compound like sodium butyrate (a fatty acid metabolite found in high concentration in the lumen of the large intestine) or a pharmacologic compound such as valproic acid (VA, a commonly prescribed antiepileptic drug), whereas others are complex chemicals isolated from culture broths of microorganisms (depsipeptide, apicidin, or trichostatin A) or synthetic derivatives (MS-275, CI-994). HDACIs are subdivided into four fundamental groups: short-chain fatty acids (sodium butyrate, phenylbutyrate, VA), synthetic benzamides derivatives (MS-275, CI-994), cyclic tetrapeptides (depsipeptide, trapoxin, apicidin), and hydroxamic acids (trichostatin A, suberoylanilide hydroxamic acid (SAHA), LAQ8240) [35]. HDACIs induce differentiation, cell cycle arrest, and/or apoptosis of cancer cells in culture and in animal models [35–37]. Multiple HDACIs (SAHA, depsipeptide, MS275) have been shown to have anticancer properties in phase I and II clinical trials [35,38–40]. The antitumor activity of HDACIs has been attributed to both their ability to inhibit deacetylases (leading to accumulation of hyperacetylated histones and alteration of gene transcription) and their ability to suppress mitogenic signal transduction pathways and downregulation of oncprotein expression [41] as well as their effect on the phenotypic expression of Bax, Bak, Bcl2, and BclXL leading to a net increase in the ratio of pro- versus antiapoptotic proteins of the Bcl2 superfamily and the apoptogenicity of the mitochondria [42–46]. It is the latter property of HDACIs that we wished to exploit to potentiate the cytotoxic effect of Apo2L/TRAIL in cultured thoracic cancer cells (cancer cell lines derived from tumors of the lung, the esophagus, or the pleura). VA, a commonly prescribed antiepileptic drug of which the pharmacokinetics and toxicity profiles are well documented [47,48], has recently been shown to be an HDACI [49–51] and to exhibit antitumor activity in vitro and in vivo animal models [52–54]. The aim of this study was to critically evaluate the cytotoxic effect of the combination of VA and Apo2L/TRAIL in a panel of cultured thoracic cancer cells. We observed that VA + Apo2L/TRAIL combination synergistically induced profound cytotoxicity and apoptosis of cultured thoracic cancer cells through the mitochondria-dependent (type II) pathway.

Materials and Methods

Cell Lines and Reagents
Cultured non–small cell lung cancer (NSCLC) cells H460 and H322; esophageal cancer (EsC) cells TE2 and TE12; and malignant pleural mesothelioma (MPM) cells H211 and H513 were maintained in RPMI 1640 culture medium supplemented with fetal calf serum (10% vol/vol), L-glutamine (1 mM), and antibiotics [streptomycin (100 µg/ml)/penicillin (100 U/ml)]. Normal human primary fibroblast and human umbilical vein endothelial cells (HUVEC) were purchased from Cambrex Bio Science (Walkerville, MD) and grown in their special culture media as per instructions of the vendor. Apo2L/TRAIL was obtained from Genentech Inc. through an institutional M-CRADA. VA was purchased from Alexis (San Diego, CA). Selective caspase 8 or caspase 9 inhibitors were purchased from R&D Systems (Minneapolis, MN). Bcl2-
overexpressing stable transductants of TE2 (TE2Bcl2) and H513 (H513Bcl2) cells were created by retrovirally mediated gene transfer using Bcl2-expressing viral vector containing green fluorescence protein (GFP) as a selectable marker (generously provided by P. Robbins, National Cancer Institute) and previously published transduction techniques [55]. Vector control stable transductants of similar cancer cells were created using GFP-expressing retrovirus. The magnitude of GFP fluorescence in Bcl2 stable transfectants closely correlated with the level of Bcl2 expression [55]. Cells with the highest level of GFP were selected by cell sorting for further culture and expansion. Bcl2 was detected using intracellular staining with phycoerythrin (PE)-conjugated anti-Bcl2 monoclonal antibody (clone Bcl-2/100, Alexis) after cell fixation and permeabilization using the Cytofix/Cytoperm kit from BD Biosciences (San Jose, CA). Flow cytometry confirmed 100% of cells expressing high levels of Bcl2. Western blot analysis demonstrated very high levels of Bcl2 in all Bcl2-expressing stable transductants.

Flow Cytometric Analysis of TRAIL Receptors
Cancer cells (controls or treated with VA at 1.0 or 5.0 mM × 24 hours) were washed with PBS with 5% BSA and incubated with biotinylated mouse antihuman antibodies for DR4, DR5, DcR1, and DcR2 (1 µg/500,000 cells; R&D Systems) for 30 minutes at room temperature. Excess unbound antibodies were removed by one wash with PBS and 5% BSA; cells were further incubated with streptavidin-PE (30 µL/500,000 cells; R&D Systems) for 30 minutes and washed once before being submitted for flow cytometry. The levels of receptor expression were quantified by the PE mean fluorescence intensity index (MFII), the ratio of PE mean fluorescence intensity of cells incubated with antireceptor antibody and the background PE mean fluorescence intensity in cells incubated with IgG isotype control and the percentages of cells gated positive for receptor expression.

Cytotoxicity and Apoptosis Assays
Cells were seeded in 96-well microtiter plates at predetermined plating densities appropriate for each cell line [(1.0 – 1.5) × 10^4 cells/well]. After an overnight incubation, cells were treated with either VA (0.5, 1, or 5 mM) concurrently with Apo2L/TRAIL (5 to 100 ng/ml; VA + Apo2L/TRAIL) or 12 hours pretreatment of VA followed by addition of Apo2L/TRAIL in the presence of VA (VA/Apo2L/TRAIL). Cell viability was quantified by (4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) at 36 hours after the onset of Apo2L/TRAIL exposure. Cell viability after Apo2L/TRAIL treatment or combinations of VA and Apo2L/TRAIL treatments were calculated as percentages of untreated controls or VA-treated controls (to normalize for the very mild growth-inhibitory effect of VA, which was less than 15% at the highest concentration of VA at 5 mM), respectively. The Apo2L/TRAIL IC_{50} values (indices of cellular sensitivity to Apo2L/TRAIL) were estimated from the respective dose-response curves. Apoptosis after treatments with Apo2L/TRAIL (10 or 20 ng/ml) alone or in combination with VA (0.5 to 5.0 mM) was determined by the terminal deoxynucleotidyldtransferase-mediated dUTP nick end labeling (TUNEL)–based Apo-bromodeoxyuridine (BrdU) assay (BD Pharmingen, San Jose, CA) for late apoptosis (48 hours after Apo2L/TRAIL exposure).

Caspase Activity Assay
Specific enzymatic activity of caspases 8, 9, and 3 at intervals after the onset of treatment with Apo2L/TRAIL (10 or 20 ng/ml) with or without VA (0.5 mM) combination was measured by a fluorometric kit (R&D Systems). Cells were treated with VA alone (0.5 mM), Apo2L/TRAIL alone (20 ng/ml), or VA + Apo2L/TRAIL combination and serially harvested at 2-hour intervals and assayed for caspase activity. The specific caspase activity, normalized for total proteins of cell lysates, was then expressed as fold of the baseline caspase activity of untreated control cells.

Western Blots
Control or VA-treated cultured thoracic cancer cells were harvested after 12 hours of VA treatment in Laemmli lysis buffer for Western blot analysis for the expression of FADD, pro-caspase 8 (BD Biosciences, 1:250 and 1:500 dilution, respectively), TRADD (Santa Cruz Biotechnology, Santa Cruz, CA, 1:200 dilution), FLIP (Alexis, 1:200 dilution), Bax, Bak, Bcl2 and BclXL, BID (all purchased from Cell Signaling Technology, Beverly, MA at 1:500 dilution), acetylated histone 3 and histone 4 (Upstate Biotechnology, Waltham, MA at 1:1000 dilution). For detection of acetylated histones, acidic cell lysates were prepared as described in the Web site of the Chromatin and Gene Expression Group headed by Prof. Bryan Turner (University of Birmingham, UK; http://medweb.bham.ac.uk/research/chromatin/protocol/extraction.html). Blots were also probed for β-actin (Oncogene Research Products, Cambridge, MA, 1:5000 dilution) to verify equal protein loadings. The primary antibodies were probed with HRP-conjugated antimouse or antirabbit secondary antibodies and detected with West Dura chemiluminescence (Pierce Biotechnology, Rockford, IL).

Statistical Analysis
Data are presented as means ± SEM of three independent experiments each performed in duplicates. Two-tailed Student’s t test was used for statistical analysis and P values less than .05 were considered statistically significant.

Results

Mild Growth Inhibitory Effect of VA on Thoracic Cancer Cells In Vitro
Valproic acid induced significant accumulation of hyper-acetylated histone protein H3 and H4 in H322, H513, or TE12 cells (Figure 1A). Continuous exposure of cultured thoracic cancer cells to VA for 96 hours led to a mild dose-dependent reduction of cell proliferation (IC_{50} values ranging from 3.2 to 5.0 mM; Figure 1A) that was mainly attributable to induction of cell cycle arrest at G1/S checkpoint (data not shown) and a weak induction of apoptosis (<15% at 5.0 mM; Figure 1B).
Synergistic Induction of Cytotoxicity and Apoptosis by VA and Apo2L/TRAIL Combinations

We next wished to determine if treating thoracic cancer cells with the combination of VA (at concentrations that are clinically achievable and have HDAC inhibitory activity) and Apo2L/TRAIL would mediate synergistic induction of cytotoxicity and apoptosis, similar to other more familiar HDACIs. Intrinsic sensitivity to Apo2L/TRAIL varied greatly between the six cultured thoracic cell lines used in this study, with H460, H322, H211, and TE12 cells being sensitive to this ligand (IC\textsubscript{50} values <100 ng/ml) and TE2 and H513 cells being more refractory to the cytotoxic effect of Apo2L/TRAIL (IC\textsubscript{50} values >150 ng/ml) (Figure 2A). The cytotoxic effect of high concentrations of Apo2L/TRAIL (100 or 200 ng/ml) in TE2, TE12, or H513 cells was totally abrogated by Bcl2, indicating that these were type II cells (Figure 2B). Concurrent exposure of cultured thoracic cancer cells to VA (0.5 to 5.0 mM) and Apo2L/TRAIL (5 to 100 ng/ml) for 36 hours resulted in significant supra-additive suppression of cell viability in MTT assays (representative data for H513, H460, and TE12 cells are shown in Figure 3A). VA alone, at the treatment conditions used, mediated little reduction of viability (15–20% at 5.0 mM). When normalized for this mild VA-mediated cytotoxic effect, there was substantial further suppression of cell viability in combination-treated cells (Figure 3A). Using Apo2L/TRAIL IC\textsubscript{50} values as indicators of cellular sensitivity to this ligand, we found that there was 1.5- to >10-fold reduction of Apo2L/TRAIL IC\textsubscript{50} values in VA-treated cells in a VA dose-dependent manner (Figure 3B). This effect was observed in all cultured thoracic...
cancer cells regardless of their intrinsic sensitivity to Apo2L/TRAIL and particularly most pronounced in Apo2L/TRAIL-resistant (TE2, TE12, and H513 cells) cell lines. Moreover, VA treatment did not affect the intrinsic susceptibility of the Apo2L/TRAIL-sensitive cells H322, H460, and H211 to this ligand. Most importantly, the VA + Apo2L/TRAIL combination, although very toxic to cancer cells, was not harmful to primary cultured normal cells (Figure 3C). Treating cancer cells with either concurrent (VA + Apo2L/TRAIL) or sequential VA followed by Apo2L/TRAIL in the presence of VA/ A po2 L/ TRAIL) schedule similarly sensitized cancer cells to Apo2L/TRAIL (Figure 3D). Concurrent VA + Apo2L/TRAIL combination profoundly and synergistically induced apoptosis in thoracic cancer cells, even at VA concentration as low as 0.5 mM (Figure 4).

The Effect of VA on Expression of TRAIL Receptors and Proapoptotic Proteins

We next sought to determine if VA increased the expression of TRAIL receptors DR4, DR5 and DcR1, DcR2, as other HDACIs have been shown to upregulate TRAIL receptor expression in cultured cancer cells of different histology [56–61]. Treating H460, H513, or TE12 cells with VA (5 mM for 12 hours) did not substantially change the basal expression of TRAIL receptors in these cells except a two-fold reduction of DR4 in H460 cells (Figure 5A). As significant cytotoxicity was observed after concurrent exposure of cancer cells to VA and Apo2L/TRAIL, and knowing that Apo2L/TRAIL would bind to its cognate receptors and initiate the signaling cascade within minutes of exposure to receptor-bearing cells, it is conceivable that alteration of TRAIL receptor repertoire by VA would not play any significant role in the process of enhancing the cytotoxic effect of Apo2L/TRAIL in VA-treated cells. We thus further investigated the effect of VA on other components of the apoptosis-inducing apparatus, namely, members of the DISC (FLIP, FADD, pro-caspase 8) and the mitochondria-associated pro- or anti-apoptotic proteins (Bax, Bak, Bcl2, BclXL). Time-course experiments indicated that there was no discernible alteration of the levels of these proteins in H513, H460, or TE12 cells continuously treated with VA (1 mM) and harvested at indicated time points after the onset of drug exposure (Figure 5B). We next turned our attention on analyzing the functional aspect of the caspase cascades in combination-treated cells.

Involvement of the Mitochondria-Dependent Death Signal Cascade in VA-Mediated Enhancement of Apo2L/TRAIL Sensitivity

The specific proteolytic activities of caspases 8, 9, and 3 were assayed at intervals after drug treatment in H513 cells (parental cells or Bcl2-overexpressing stable transfectants H513Bcl2 cells) after exposure to VA, Apo2L/TRAIL, or sequential VA/Apo2L/TRAIL combination. Whereas no or little (zero to four-fold) activation of caspases 8, 9, or 3 was observed after exposure of H513 cells to VA (0.5 mM) or Apo2L/TRAIL (20 ng/ml), a supra-additive five- to eight-fold increase in the activity of these caspases was observed in cells treated with the VA + Apo2L/TRAIL combination. More interestingly, treatment-induced activation of not only caspase 9 but also of caspase 8 and caspase 3 was totally abrogated by overexpression of Bcl2 (Figure 6). Similarly, complete suppression of the high levels of caspases 8, 9, and 3 was noted in similarly treated cells incubated with the selective caspase 9 inhibitor Z-LEHD-fmk (data not shown). In both experiments, inhibition of caspase 9 activation served as an internal control, whereas complete inhibition of caspase 3 indicated the exclusive role of the mitochondria pathway in activating this downstream executioner caspase, and inhibition of caspase 8 implied activation of this apical caspase was downstream of caspase 9 and the result of the amplification feedback loop mediated by the mitochondria-dependent caspase activation cascade.

Bcl2-mediated inhibition of caspases 8, 9 and 3 activity was translated to complete abrogation of the intense cytotoxic effect of the VA + Apo2L/TRAIL combination in Bcl2 stable transfectants H513Bcl2 and TE12Bcl2 (Figure 7A). The mild growth-inhibitory effect of Apo2L/TRAIL (20% or 30% reduction of cell viability in TE12 or H513, respectively) was
also sensitive to Bcl2 overexpression, indicating that these were type II cells. The essential role of the mitochondria-mediated death signal cascade in combination-treated cells was further evidenced by the complete abrogation of apoptosis by the selective caspase 9 inhibitor (Figure 7B).

Discussion
Primary cancers of the thoracic cavity (cancers of the lung, the esophagus, or the pleura) when presented with regional or systemic metastasis are notoriously refractory to standard-of-care therapy regimes of cytotoxic chemotherapy, external beam irradiation, and surgical resection in various combinations [62,63]. Better understanding of the molecular basis of carcinogenesis and elucidation of signal transduction pathways regulating cell growth and death in normal cells and their roles in the process of malignant transformation offers great opportunities for the development of novel, molecularly targeted anticancer therapy. Within this context, therapeutic strategies aiming at direct induction of cell death by activation of the TRAIL receptor-mediated signal transduction pathways has attracted a great deal of attention and, in fact, recently entered early-phase clinical development. Recombinant protein (such as Apo2L/TRAIL [19] or recombinant human agonistic TRAIL-R1 monoclonal antibody [64] are commonly used to activate TRAIL receptor for the induction of apoptosis of cancer cells. It became clear to investigators in this field that a significant proportion of cultured malignant cells, although expressing the functional TRAIL receptors DR4/DR5, are refractory to the cytotoxic effect of recombinant soluble TRAIL [21]. The molecular basis of this intrinsic or acquired resistance to TRAIL-induced cytotoxicity in various cancer cell lines is complex and multifactorial [21,22]. Moreover, in vitro experimental conditions can significantly influence the intrinsic susceptibility of cultured cancer cells to TRAIL, thus making determination of cellular sensitivity to this death-inducing ligand or others like FasL somewhat arbitrary and subjected to a wide range of variability between laboratories. We defined Apo2L/TRAIL-sensitive cells in our study using the described

![Diagram of Figure 3](image-url)

Figure 3. (A) Dose-dependent enhancement of Apo2L/TRAIL-mediated cytotoxicity by VA (0.5 to 5 mM) in representative cultured thoracic cancer cells H460, TE12, and H513. Cell viability is expressed as percentages of viable cells in untreated controls (for Apo2L/TRAIL treatment alone) or cells exposed to VA alone (in VA + Apo2L/TRAIL–treated cells to normalize for the very mild growth-inhibitory effect of VA). Data are presented as means ± SEM of four independent experiments. (B) Lack of VA + Apo2L/TRAIL–induced cytotoxicity in either primary normal skin fibroblasts or HUVEC. Significant accumulation of acetylated H4 histone protein was observed in these primary normal cells treated with VA (1.0 or 5.0 mM for 24 hours). Data are presented as means ± SEM of three independent experiments. (C) Apo2L/TRAIL IC50 values of cultured cancer cells treated with the VA + Apo2L/TRAIL combinations. These values were estimated from the respective dose-response curves and used as indicators of cellular sensitivity to Apo2L/TRAIL. Data are presented as means ± SEM of four independent experiments. (D) Treatment schedules (concurrent VA and Apo2L/TRAIL or VA pretreatment for 12 hours before adding Apo2L/TRAIL) have no impact on the enhancement of cellular sensitivity to Apo2L/TRAIL in cell treated with drug combinations. Data are presented as means ± SEM of four independent experiments.
Profound and synergistic dose-dependent induction of apoptosis by the VA + Apo2L/TRAIL combinations in representative cultured thoracic cancer cells. Data are presented as means ± SEM of four independent experiments; #P < .001 versus Apo2L/TRAIL alone or VA treatment alone.
and antiapoptotic proteins of the Bcl2 superfamily was also observed in HDACI-treated cells and thought to play a role in sensitizing cultured cancer cells to the apoptosis-inducing effect of TRAIL [61,70,71]. Others have demonstrated the involvement of the intrinsic pathway in mediating enhanced cytotoxicity after treatment with the HDACI + TRAIL combination [61,67]. In direct contrast to previously mentioned publications, we did not observe any alteration of DR4/DR5 expression in VA-treated cells. As profound cytotoxicity was observed in cells concurrently exposed to TRAIL and HDACI and HDACI-induced upregulation of DR4/DR5 expression occurred after latent periods ranging from 4 to 6 hours in many reported studies, it is hard to attribute receptor upregulation as having a direct impact on the enhancement of cellular sensitivity to TRAIL. Even if there was a delayed upregulation of TRAIL receptors in VA-treated cells that was not readily detected by the flow cytometry method that we used in our study, such a change would not have an impact on the enhanced cytotoxicity of this drug combination, as similar degrees of growth inhibition were observed in cancer cells treated with either concurrent VA + Apo2L/TRAIL or sequential VA/Apo2L/TRAIL combinations. Enhancement of Apo2L/TRAIL tumoricidal activity by VA, either in ligand-sensitive or -resistant cells, was totally abrogated by either Bcl2 over-expression or by the selective caspase 9 inhibitor, implying that the intrinsic pathway was essential in regulating this process. Data from the caspase activity experiments defined the regulatory role of the mitochondria-derived caspase 9 (and possibly caspase 3) in amplifying the caspase cascade by potentiating the activation of caspase 8 after Apo2L/TRAIL exposure, particularly in the presence of VA. The strong caspase 8 activation in combination-treated H513 cells may be the result of increased DISC activity or secondary to activation by downstream executioner caspases such as caspase 3. Processing of caspase 3 can be mediated by caspase 8 (extrinsic pathway) or by caspase 9 (intrinsic pathway). The hierarchical ordering of caspase activation downstream of caspase 9 has been well described [72]. Caspases 3, 8, 7, and 10 are all substrates of activated caspase 9; whether caspase 9 directly processes caspase 8 is not completely clear, but it is well known that caspase 3 does activate caspase 8. It is entirely possible that, via caspase 3, caspase 9 indirectly regulates caspase 8 activation. Blocking of caspase 3 activity using a selective inhibitor would abrogate caspase 8 activation if this was indeed secondary to downstream caspase-dependent feedback loop, but caspase 3 blockade would not discriminate which pathway downstream from the DISC (intrinsic versus extrinsic) is responsible for perpetuation of caspase 8 activation. However, inhibition of caspase 9 activation by using the selective inhibitor Z-LEHD-fmk or overexpression of Bcl2 would block both caspase 3 and caspase 8 activation if proteolytic processing of caspase 8 was secondary to caspase 3 activation through the mitochondria-mediated intrinsic pathway. This was exactly what we observed in that caspase 8 activation in combination-treated cells was completely suppressed by either Bcl2 overexpression or the selective caspase 9 inhibitor. VA, therefore, through a molecular mechanism yet to be
fully elucidated further, activates the intrinsic pathway of type II cells to increase the efficacy of Apo2L/TRAIL. As we have not identified type I cells in our panel of cultured thoracic cancer cells, it is not known if VA would activate the intrinsic signaling cascade to induced Apo2L/TRAIL sensitization, thus invoking the type II characteristic to potentiate the cytotoxicity of this ligand in such cell lines. Our attempt to evaluate the effect of VA on altering phenotypic expressions of key DISC proteins or some well-described mitochondria-related pro- and antiapoptotic proteins was not fruitful. This was in contrast to previous findings of other investigators as well as of our own observation of profound upregulation of Bax and/or Bak in conjunction with reduction of the levels of Bcl2/BclXL shortly after treating the same cancer cells with a more potent HDACI trichostatin A (Reddy et al., unpublished data). Functionally, we made similar observations with VA + Apo2L/TRAIL combinations but were unable to reproduce the phenotypic alterations of members of the Bcl2 superfamily after VA treatment. Ongoing studies are being conducted to define the molecular mechanism by which VA instigates the mitochondria to sensitize cancer cells to Apo2L/TRAIL.

In summary, VA, at clinically achievable drug concentrations, profoundly enhances the intrinsic sensitivity of cultured thoracic cancer cells to Apo2L/TRAIL in vitro. As VA is a Food and Drug Administration–approved antiepileptic drug with well-described pharmacokinetics and toxicity profiles and Apo2L/TRAIL is currently undergoing clinical testing, this drug combination has a good translational potential and should be considered for clinical development.

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Figure 7. (A) Overexpression of Bcl2 totally suppressed apoptosis induced by VA + Apo2L/TRAIL combinations in cultured thoracic cancer cells. Parental or Bcl2-overexpressing stable transfectants H513Bcl2 and TEBcl2 were concurrently treated with VA (5.0 mM) and apo2L/TRAIL (20 ng/ml). Cell viability was evaluated by MTT assay. Vector controls behaved exactly like parental cells. Data are presented as means ± SDs of four independent experiments. (B) Profound inhibition of VA + Apo2L/TRAIL–induced apoptosis by the selective caspase 9 inhibitor Z-LEHD-fmk (40 μM). Cells were pretreated with the selective caspase 9 inhibitor for 1 hour before addition of VA (5.0 mM) and Apo2L/TRAIL (10 or 20 ng/ml). Cells were harvested 48 hours later and apoptosis was determined by the TUNEL-based Apo-BrdU assay. Representative data of three independent experiments with similar results are shown here.
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