Valproic Acid Sensitizes K562 Erythroleukemia Cells to TRAIL/Apo2L-induced Apoptosis

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Abstract. *Background: Selectively targeting death receptors to trigger apoptosis in cancer cells appears ideal in cancer therapy. The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo2L) is of great interest since it has been shown to predominantly kill cancer cells without toxic effects on normal counterparts, thus representing a promising anticancer agent. However, resistance towards TRAIL/Apo2L treatment has also been described. To overcome this obstacle, co-administration of TRAIL/Apo2L plus several compounds, including histone deacetylase inhibitors (HDACi), has been attempted as a strategy to restore cancer cell sensitivity to TRAIL-induced apoptosis. In recent years, the clinical application of HDACi has been largely explored for their ability to modulate gene transcription, block cell division cycle, inhibit cell proliferation, induce cellular differentiation and apoptosis. Materials and Methods: The ability of valproic acid (VPA), a well-known HDACi, to sensitise the K562 cell line, derived from a human leukemia, to TRAIL/Apo2L-mediated apoptosis was evaluated. VPA was selected since it is currently used in clinical practice and its pharmacokinetic, pharmacodynamic and bioavailability are known. Results: When applied with TRAIL/Apo2L, VPA increased cell death and caspase-3 activity by 4-fold compared to the treatment with TRAIL/Apo2L alone. VPA sensitized K562 cells to TRAIL/Apo2L-mediated apoptosis by increasing the expression of DR4 and DR5 by 3- and 14-fold respectively. In addition, VPA per se, in the absence of TRAIL/Apo2L, reduced the expression of antiapoptotic factors, such as c-FLPs, associated with DISC, and Bcl-2/Bcl-X_L, associated with mitochondria, acting on both extrinsic and intrinsic apoptotic pathways. Conclusion: Our results demonstrated the ability of VPA to sensitize TRAIL/Apo2L-resistant cells to apoptosis, thus providing an attractive approach for the treatment of leukemias and other proliferative malignancies.*

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Apoptosis is an active form of programmed cell death, acting in concert with other important biological processes, such as cell division, immune responses, development and tissue homeostasis (1). Apoptosis can be induced following stimulation of different types of cell death receptors (DRs), including tumor necrosis factor (TNF) receptor I Fas/CD95, and the TNF-related apoptosis-inducing ligand (TRAIL/Apo2L) receptors DR4 and DR5 (also known as TRAIL-R1 and TRAIL-R2, respectively). Stimulation of DRs by their specific ligands FasL, TNF-alpha and TRAIL/Apo2L causes, in reactive cells, oligomerization of the receptor and activation of complex death machinery resulting in DNA fragmentation and disruption of normal cellular and nuclear morphology (2).

TRAIL/Apo2L has gained interest as a promising agent in cancer therapy (3-4) since the observation that TRAIL/Apo2L predominantly kills cancer cells without affecting normal cells (5-11). TRAIL/Apo2L is expressed in various cells of the immune system and is involved in both T-cell- and natural killer cell-mediated tumor surveillance, and in suppression of suppressing tumor metastasis (2). TRAIL/Apo2L is capable of initiating apoptosis through engagement of its related DRs. Similarly to other TNF family members, TRAIL forms homotrimers that bind three receptor units. In contrast to other TNF family members, TRAIL mRNA is constitutively expressed in many tissues (12). Although the main biological function of TRAIL/Apo2L has been associated with apoptosis induction, the complete physiological role of the ligand has not been fully investigated. TRAIL/Apo2L-deficient mice are more prone to develop experimental and spontaneous tumor metastasis than mice expressing TRAIL/Apo2L, suggesting that the ligand is part of a surveillance system responsible for the elimination of developing tumors (13). However, the promising demonstration that TRAIL/Apo2L treatment induced regression of established tumors in a mouse model (7) was followed by data reporting that the same molecules also killed normal brain and liver cells (8-

14). Currently, clinical trials are in progress to test the ability of different formulations of TRAIL/Apo2L ligands, and humanized anti-TRAIL/Apo2L antibodies to trigger DR4 and DR5 (15).

Resistance towards TRAIL/Apo2L has been also reported in about 50% of screened cancer cell lines (16). A plethora of compounds, including standard chemotherapeutic drugs, when co-administered with TRAIL/Apo2L, have been reported to be able to sensitize tumor cells to TRAIL/Apo2L-induced apoptosis (17-19) without changing the apoptosis resistance barrier of untransformed cells (17), suggesting that TRAIL/Apo2L efficiency might be further increased by combining it with low doses of standard chemotherapy (20-21). Thus, the solution to the clinical use of Apo2L/TRAIL may reside in basing combined regimens on bypassing resistance.

In recent years, chromatin remodelling actions have emerged as the basis for gene regulation, and aberrant architecture of the chromatin has been implicated in cancer formation and progression (22). Histones are subject to post-translational events including acetylation, methylation, phosphorylation, and ADP ribosylation (23-24). Dynamic modulation of chromatin structure/function is mainly mediated by acetylation and deacetylation reactions of ε-amino groups of lysine residues present in core histones. The enzymes responsible for these processes are known as histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively (25). It has been shown that HATs usually act as transcriptional coactivators, while HDACs are part of transcriptional co-repressor complexes. Treatment with HDAC inhibitors (HDACi) produces reversible hyperacetylation of core histones as a consequence of HDAC inhibition. HDACi have shown generalized therapeutic potential since they block the cell division cycle, inhibit cell proliferation and induce cellular differentiation and apoptosis (26-27). These effects have been confirmed in both *in vitro* and *in vivo* experimental models including acute promyelocytic leukemias and cell lines derived from solid tumors such as colon, lung, neuroblastoma, glioma, teratocarcinoma, prostate carcinomas and others (28). As a consequence, several molecules classified as HDACi are currently employed in clinical trials (29) and, recently, the FDA approved the drug SAHA (suberoylanilide hydroxamic) to treat cutaneous T-cell lymphoma. HDACi are potential candidates for increasing TRAIL/Apo2L efficacy in combined therapy; in fact, their activity in sensitizing human cancer cells to TRAIL/Apo2L-induced apoptosis was recently established in a number of human tumors (30-31).

Valproic acid (VPA) is a branched short-chain fatty acid long employed as an anticonvulsant. In fact, the molecule is used in medical practice to treat a variety of neurological disorders, as well as depressive illness, bipolar disorder and epilepsy (32), therefore, its pharmacokinetic, pharmacodynamic and bioavailability are well established. More recently, VPA has been shown to alter the acetylation state of the core histones by a mechanism involving the inhibition of the HDAC enzymes (33).

There are two main pathways by which apoptosis is activated: the extrinsic and intrinsic pathways. The first is activated by the engagement of death receptors on the cell surface and the consequent formation of the death induced signalling complex (DISC). DISC in turn recruits caspase-8 and promotes the cascade of procaspase activation that follows (34). The intrinsic pathway is triggered by various extracellular and intracellular stresses, which converge mainly on the mitochondria, and results in the permeabilization of the outer mitochondrial membrane, the release of cytochrome *c*, the formation of the apoptosome and caspase activation (35). The key permeabilization step is regulated by the antiapoptotic members of the Bcl-2 family (36). c-FLIP (FLICE inhibitory protein) is a DISC member, homologous to caspase-8, and exists as two alternatively spliced isoforms. The majority of the studies have recognized the c-FLIP *short form* (c-FLIPs) as a potent suppressor of the apoptotic pathway (37). In fact, c-FLIPs may be recruited to the activated DR *via* FADD, thereby either preventing the enrolment of procaspase 8 to the DISC, or inhibiting the induced activation of caspase-8, interfering with signalling from the DISC (38).

The aim of this study was to evaluate the ability of VPA to sensitise human K562 leukemia-derived cells to TRAIL/Apo2L-mediated apoptosis. The novelty of this work resides in the observation that VPA restores sensitivity to TRAIL/Apo2L-mediated cell death by acting at different levels on both intrinsic and extrinsic apoptotic pathways.

Materials and Methods

Cell culture, reagents and treatments. The human erythroleukemia K562 cell line was obtained from the ATCC (Philadelphia, USA). Cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum, penicillin/streptomycin and 2 mM L-glutamine. All experiments were performed using cells in logarithmic phase growth suspended at $0.5x10^5$ cells/ml. Cells were cultured in the presence of different concentrations (1-50 ng/mL) of recombinant human TRAIL/Apo2L (Super Killer TRAIL), purchased from Alexis Biochemicals (Vinci-Biochem, Firenze, Italy). Viable cell counts were determined using trypan-blue dye exclusion test and/or propidium iodide (PI) incorporation (39). Valproic acid (VPA), butyric acid (BT), hydroxyurea (HYU), PI and benzidine were from Sigma-Aldrich (Milan, Italy). Daunorubicin (Daunoblastin™; Pharmacia, Erlangen, Germany) was tested at final concentration of 0.4 μg/mL.

Benzidine hemoglobin staining. Cells containing hemoglobin were detected using benzidine/hydrogen peroxide solution as described elsewhere (40). Briefly, a solution containing 0.2% benzidine in

0.5 M acetic acid was added, just before use, with 20 μl/mL of a 3% hydrogen peroxide solution to an equal volume of cell suspension in 0.9% NaCl. Cell dilutions were analyzed under a Zeiss Axiovert 200 microscope. Cells appeared clearly positive or negative without intermediate degrees of staining.

SDS-polyacrylamide gel electrophoresis and Western blotting. Proteins were analysed from control and treated cells. Cells were harvested by centrifugation and washed once with phosphate-buffered saline (PBS) then lysed in lysis buffer [50 mM Tris/HCl, (pH 7.4), 500 mM NaCl, 1% Nonidet P-40, 10 mM EDTA, 10% glycerol, 0.5 mM dithiothreitol] containing protease inhibitors (100 mg/L phenylmethylsulfonyl fluoride, 100 mg/L tosyl-phenyl-chloromethyl ketone, 1 mg/L leupeptin, 0.83 mg/L chymostatin, 10 mg/L soybean trypsin inhibitor, 1 mg/L pepstatin). Total protein (25 μg) was loaded on a 12% SDS polyacrylamide gel and transferred to polyvinylidene fluoride membranes (Millipore, Rome, Italy) and subsequently incubated with commercially available antibodies. Anti-DR4, -DR5, poly(ADP-ribose)polymerase (PARP), Bcl-X_L and FLICE inhibitory protein (FLIP) were from Santa Cruz Biotechnology (Heidelberg, Germany); anti-caspase 8 was from Alexis Biochemicals (Milan, Italy); α-tubulin was from Sigma-Aldrich; anti-Bcl-2 was fom Calbiochem (Milan, Italy); horseradish peroxidase-conjugated secondary antibodies were purchased from GE Healthcare Bio-Sciences (Milan, Italy). Immunoreactivity was visualised using chemiluminescence reagents according to the manufacturer's instructions (ECL plus; GE Healthcare Bio-Sciences).

Caspase-3 activity assay. Enzymatic activity of caspase-3 was determined, as previously described (41-42), 24 hours following treatment with 20 ng/ml TRAIL/Apo2L ligand. Briefly, cell pellets were suspended in 60 μl of lysis buffer [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes, pH 7.4), 2 mM ethylenediamine tetra-acetic acid (EDTA), 0.2% CHAPS, 5 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride], centrifuged at 13,000 xg, and the protein concentration was determined. Subsequently, 20 μg of supernatant were assayed in caspase-3 assay buffer containing in a total volume of 100 μl: 100 mM Hepes (pH 7.4), 20% v/v glycerol, 0.5 mM EDTA, 5 mM dithiothreitol, and 1 μl of caspase-3 substrate (Ac-DEVD-AFC; Alexis Biochemicals). After incubation for 30 min at 37˚C, proteolytic cleavage of the substrate freed the fluorochrome (AFC) that was detected spectrofluorimetrically with excitation and emission settings at 395 and 540 nm, respectively.

Caspase-3 specific activity was calculated as nmoles AFC/min/μg of total protein.

Quantitative PCR analysis of DR4 and DR5 receptors. DR4 and DR5 mRNA levels were determined by quantitative PCR (qPCR). Total RNA was obtained by guanidinium/phenol:chloroform procedure using Trizol reagent (Invitrogen, Milan, Italy) following the manufacturer's instructions. Reverse transcriptions were performed on 2 μg total RNA in a reaction volume of 20 μl containing 4 U of Omniscript-RT (Qiagen, Milan, Italy), 2 μl of 10x RT buffer, 1 μM Oligo-dT primer (Invitrogen), 2 mM dNTP mix (GE Healthcare Bio-Sciences), 4 U RNasin ribonuclease inhibitor (Promega, Milan, Italy). Reactions were incubated for 60 minutes at 37˚C before volume adjustment to 200 μl in TE buffer. SYBR green-labeled PCR amplifications were performed following the manufacturer's instructions employing an ABI Prism 7000 SDS real-time thermal cycler (Applied Biosystems, Milan, Italy). cDNA Templates were added in 50 μl final volume reaction containing Platinum SYBR Green qPCR SuperMix UDG (Invitrogen). ROX reference dye was included in the SuperMix to normalize the fluorescent signals.The amplification protocol included 2 minutes at 50˚C to activate the incorporated uracil-N-glycosylase (to prevent the reamplification of any carryover PCR products), 2 minutes at 95˚C, 40 cycles of 15 seconds at 95˚C for denaturation, and 60 seconds at 60˚C for annealing and extension. The sequences for the sense and antisense primers for DR4 and DR5 were published elsewhere (43). The sequences of the sense and anti-sense primers for the housekeeping glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript were S-5'-ATGACATCAAGAAGGTGGTG-3' and AS-5'-CATACCAGGAAATGAGCTTG-3', respectively. Specificity of formed PCR products was confirmed using melting curve testing and agarose gel electrophoresis (data not shown). Expression data obtained from qPCR were normalized to GAPDH using the comparative Ct method ($2^{-\Delta\Delta Ct}$) (44). Using this method, an average fold-change in gene expression was obtained for each transcript.

Surface expression analysis of DR4 and DR5 receptors. K562 cells ($1x10^6$) were treated with 1 mM VPA for 48 hours. After incubation, cells were washed twice in PBS, resuspended in blocking buffer (10% normal rabbit serum in PBS/0.2% sodium azide) and incubated for 30 minutes on ice to block nonspecific binding, then washed and incubated with 1 μg of anti-DR4 or anti-DR5 specific antibodies (Alexis Biochemicals) in 1% bovine serum albumin in PBS-azide for 30 minutes, followed by washing in PBS. Samples without primary antibody treatment were used as controls. Finally, cells were incubated with secondary antibody FITC-conjugated (Sigma-Aldrich) for 30 minutes, and after washes, were analyzed on a FACScalibur flow cytometer (BD Biosciences-Clontech, Milan, Italy) as described elsewhere (45).

Statistical analysis. Each experiment was performed at least three times and all values are represented as means±SD of triplicates. Student's *t*-test was used to analyze a statistical significance of the results. Values of $p<0.05$ were considered as statistically significant.

Results

VPA induced K562 cell differentiation. The effects of VPA on cell viability and differentiation were evaluated. For this purpose we selected the bcr/abl-positive erythroleukemia K562 cell line that can be induced to differentiate toward the erythroid lineage by treatment with different compounds including BT and HYU (46-47). Moreover, K562 cells were selected for their known resistance to DR-induced apoptosis (38).

To investigate whether VPA affects K562 cells viability, a trypan blue exclusion test was performed (Figure 1). Cells were exposed to increasing concentrations of VPA for 24, 48 and 72 hours. Cell viability was reduced by all concentrations and all incubation periods to the same extent. Therefore, for the subsequent experiments, a concentration of 1 mM VPA was selected. Treating K562 cells with 1 mM VPA for 72 hours, clear erythrocytic

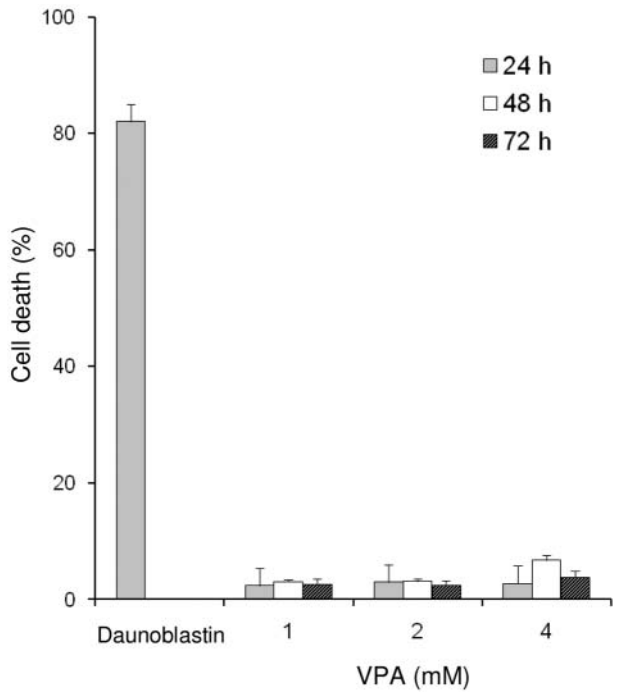


Figure 1. *Effects of VPA on K562 cell viability. Cells were treated with increasing concentrations of VPA for 24-72 hours. Numbers on the Y-axis represent the proportion of viable cells as a percentage of the control cells as evaluated by trypan-blue staining. VPA exerted only minor effects on K562 cell viability at the concentrations assayed.*

differentiation was induced, as shown by the increased expression of fetal hemoglobin evaluated by the fraction of benzidine-positive cells (Figure 2). Similar data were obtained after 48 hours' incubation in the presence of 1 mM VPA (data not shown). The ability of VPA to induce hemoglobin production appeared comparable to other differentiating agents, such as BT and HYU employed as positive controls (Figure 2, panels C and D, respectively).

Combination of VPA and TRAIL increased cell death. HDACi are usually known to induce growth arrest, differentiation and/or apoptosis (29). Whether VPA would affect cell sensitivity to DR-mediated apoptosis was investigated. The K562 cell line, as described, is relatively resistant to apoptosis induced by DRs, such as FasL, TNF-α, and TRAIL/Apo2L (38). However, several studies indicate that DR responses may be strictly regulated by erythroid differentiation (38). Preliminarily, the effect of TRAIL (1-50 ng/mL) on K562 cell viability for 24 hours was evaluated by flow cytometry through a PI exclusion test. As expected, K562 cells exhibited a low sensitivity to TRAIL-induced apoptosis (Figure 3). A slight increase in cell death (12%) was found at 20 ng/mL TRAIL. At higher concentrations of the ligand, we did not observe any increase in cell death. Based on these experimental data, a concentration of 20 ng/mL TRAIL ligand was used in combinatory treatments with VPA.

Firstly, cells were co-incubated with 20 ng/mL TRAIL plus 1 mM VPA for 24 hours and cell death measured by PI incorporation. The data shown in Figure 4A (white bars) indicates that the combination resulted in a synergistic reduction of K562 cell viability of approximately 30% compared to the treatment with TRAIL alone. The effect was even enhanced (>50% cell death) when a sequential treatment was carried out on K562 cells: 24 hours pre-incubation with 1 mM VPA followed by 24 hours incubation in the same medium with 20 ng/ml TRAIL added (Figure 4A, grey bars). This experimental protocol was adopted in the subsequent experiments.

To assess if the increase in K562 cell death was associated with an active apoptotic process, the effect of VPA plus TRAIL incubation on the activity of caspase-3, an effector caspase in the apoptotic process, was measured. As shown in Figure 4B, a 3-fold increase in caspase-3 activity was detected after VPA/TRAIL combined treatment. Apoptosis was also established by increased procaspase-3 cleavage after VPA/TRAIL treatment (Figure 4C). The cleavage of PARP-1 (poly(ADP-ribose) polymerase-1) (48) leading to PARP inactivation by caspase-3 is important for turning off an energetically expensive DNA repair pathway and for maintaining ATP levels that are required for the execution of apoptosis (49). Figure 4D clearly shows that the cleavage of PARP-1 perfectly matched the increased activation of caspase-3 following VPA plus TRAIL incubation.

VPA induced up-regulation of DR4 and DR5. Sensitization to TRAIL/Apo2L by HDAC inhibitors other than VPA (*e.g.* BT and trichostatin A) has previously been linked to up-regulation of TRAIL-R1 and/or TRAIL-R2 expression in a Sp1- or NF-κB-dependent manner (50-55). Since the VPA effects on gene expression are partially dependent upon Sp1 activation (56), we hypothesized that VPA might synergize TRAIL/Apo2L-dependent cell death by increasing surface expression of DRs in K562 cell. Therefore, we immunolabeled DR4 and DR5 receptors employing monoclonal antibodies and analyzed their surface expression by flow cytometry. As shown in Figure 5A, treatment with 1 mM VPA for 48 hours resulted in different up-regulation of both DR4 and DR5 receptors compared to untreated control cells. Quantitative PCR analysis demonstrated that the up-regulation of both receptors was due to an increased transcriptional activity of their genes at least. In fact, as shown in Figure 5B, a 3- and 14-fold increase in the mRNA level of DR4 and DR5, respectively, were measured. Since it was previously reported that K562 cells do not express decoy receptors for TRAIL (38), their potential involvement were not investigated.

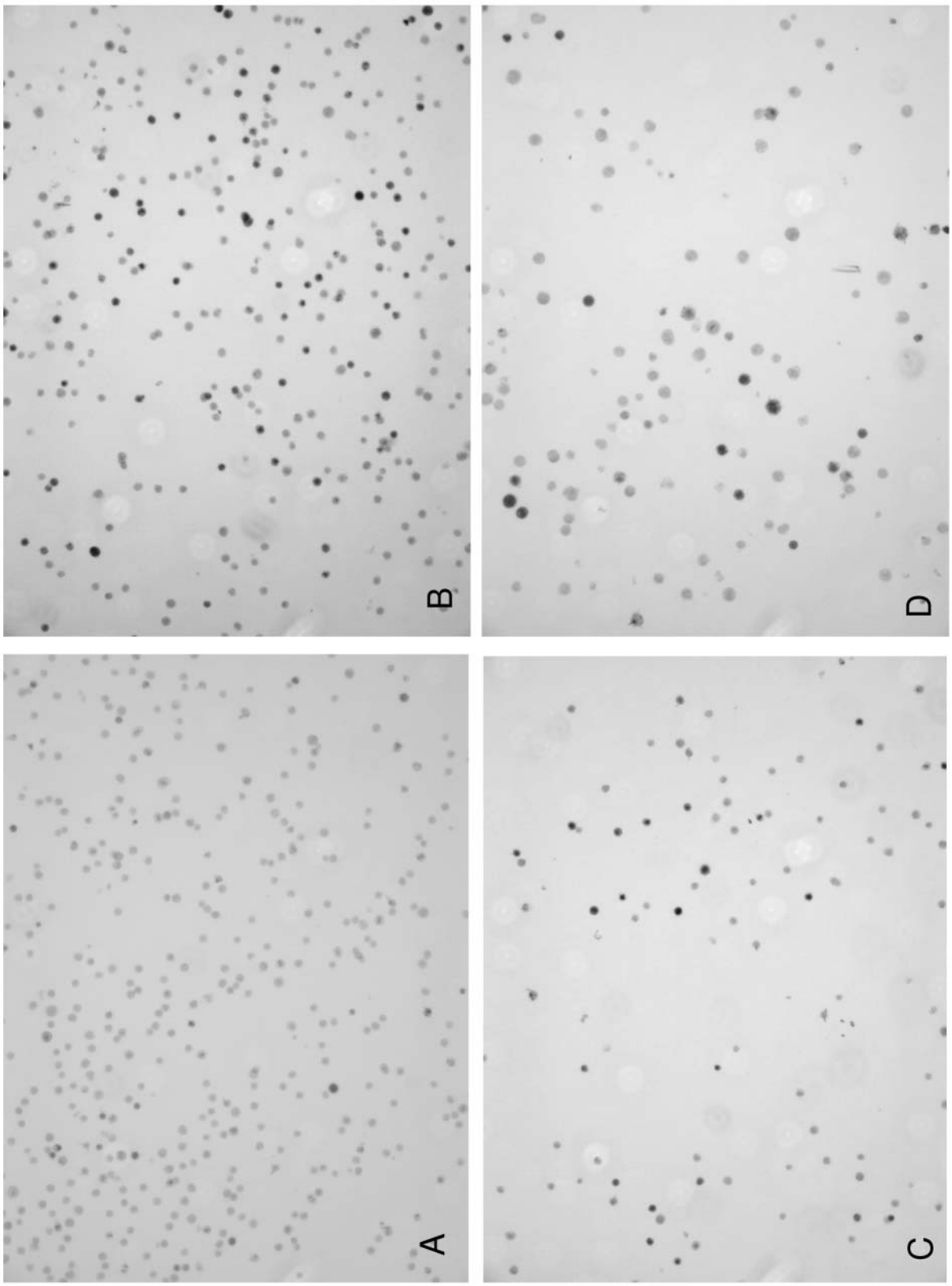


Figure 2. *K562 can be induced to differentiate toward the erythroid lineage by treatment with various compounds, including BT and HYU. Cells were incubated alone (A) or in the presence of 1 mM VPA (B), 1 mM BT (C), or 1 mM HYU (D) for 72 hours. Differentiated K562 cells containing hemoglobin were detected by benzidine/hydrogen peroxide staining. Cells dilutions were analyzed in bright field microscopy, and clearly appeared positive or negative without intermediate degrees of staining.*

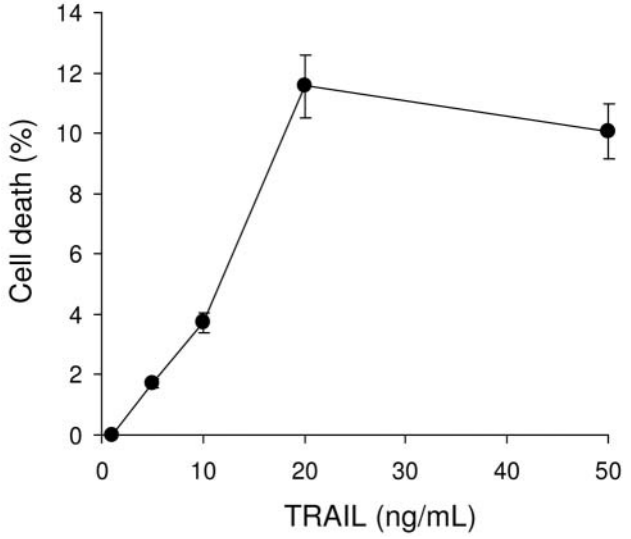


Figure 3. *Effects of TRAIL on K562 cell death. Cells were incubated in the presence of increasing concentrations of TRAIL for 24 hours and cell death was evaluated by PI exclusion assay.*

VPA sensitized both extrinsic and intrinsic apoptotic pathways. We investigated the effect of VPA on both extrinsic and intrinsic apoptotic pathways. We showed that c-FLIPs was down-regulated in VPA-treated K562 cells, concomitantly with the observed sensitization to TRAIL-induced apoptosis (Figure 6A). It is worthwhile noting that procaspase 8 cleavage did not occur in cells treated with VPA alone, but only after the combination with TRAIL (data not shown).

The involvement of VPA in the intrinsic pathway was evaluated by measuring the level of expression of mitochondria-associated antiapoptotic proteins, such as Bcl-2 and Bcl-X_L. Immunoblotting analyses showed that VPA plus TRAIL treatment in K562 cells sensitized the mitochondrial apoptotic pathway. In fact, a slight but clearly detectable reduction in the expression of both anti-apoptotic Bcl-2 and Bcl-X_L proteins was observed in VPA-treated cells (Figure 6), although this effect was not sufficient to induce apoptosis. Only when K562 cells were co-treated with VPA in combination with TRAIL was apoptosis induced.

Taken together, our data indicate that VPA sensitized K562 cells to TRAIL/Apo2L-induced apoptosis by acting on factors regulating signalling cascades of both the extrinsic and intrinsic apoptotic pathways.

Discussion

The aim of this study was to investigate the pleiotropic mechanism(s) by which VPA enhances apoptosis induced by TRAIL/Apo2L in apoptotic-resistant cell lines. Therefore, we selected the human K562 leukemia cell line, since it

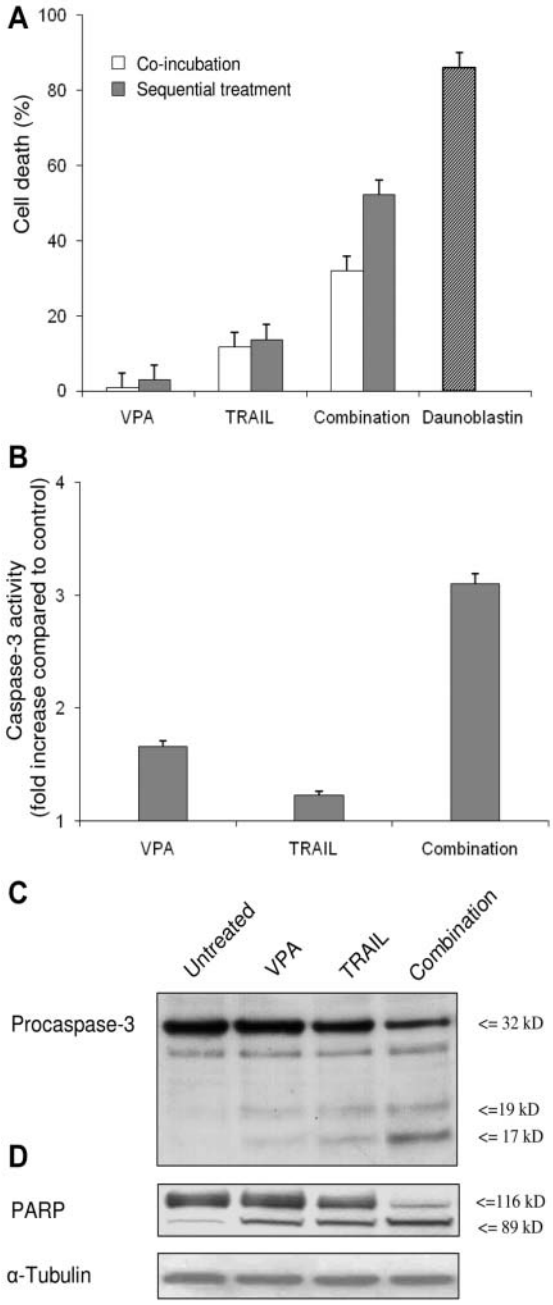
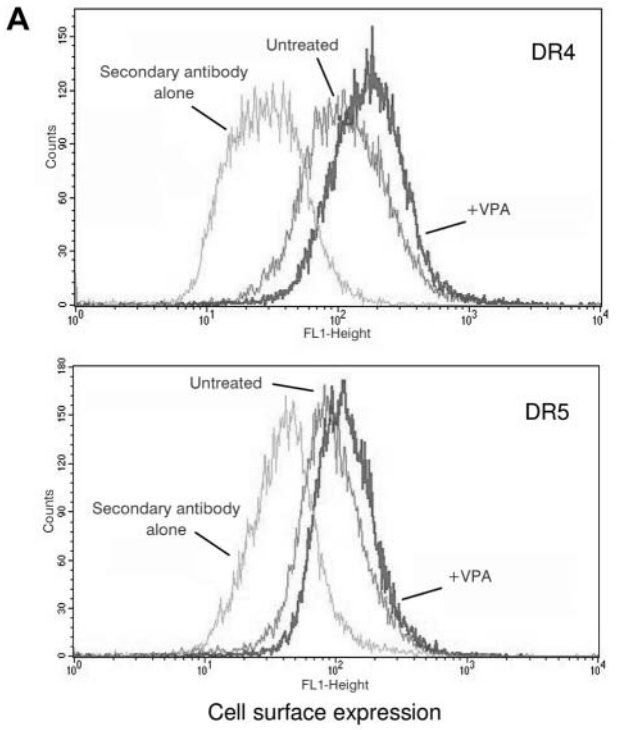


Figure 4. *(A) Effects of VPA and TRAIL, alone and in combination on K562 cell viability. Cell viability was detected as described in Material and Methods. The effects of 24 hours' co-incubation of cells with 1 mM VPA and 20 ng/mL TRAIL were assayed and compared to the effects of each drug alone (white bars). Grey bars show the effects of sequential treatment compared to singly: 24 hours pre-incubation with 1 mM VPA followed by 24 hours incubation in the same medium with 20 ng/ml TRAIL added. The daunoblastin (0.4 μg/mL) was employed as positive control. (B) K562 cells were treated with 1 mM VPA and 20 ng/mL TRAIL in sequential combination. Lysates from treated cells were used to measure the catalytic activity of caspase-3 using a fluorogenic substrate. VPA treatment significantly increased the catalytic activity of caspase-3. Catalytic activation of caspase-3 in sequentially treated cells was confirmed by enhanced cleavage of procaspase-3 (Panel C) and by the cleavage of PARP (Panel D).*



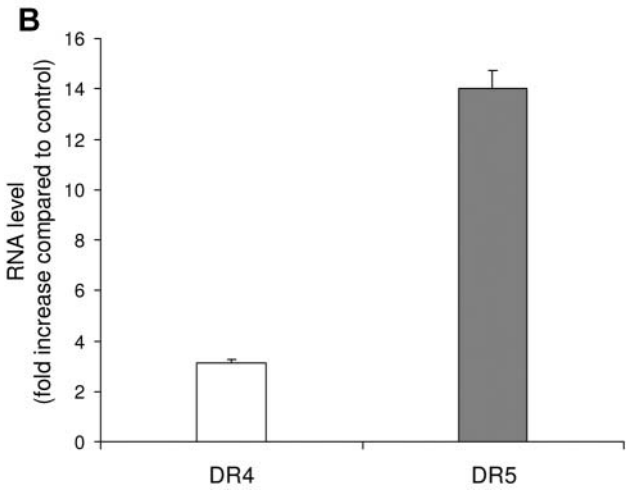


Figure 5. *VPA increased the surface expression and transcription of DR4/DR5 receptors in K562 cells. (A) Cells were treated with 1 mM VPA for 48 hours, immunolabelled against the respective receptors, and the surface expression of DR4 and DR5 was then measured by cytofluorimetric analysis as described under Materials and Methods. (B) Cells were exposed to the presence of 1 mM VPA for 48 hours. Total RNA was extracted to perform quantitative PCR as described under Materials and Methods. GAPDH was used as internal control. Results were expressed as fold increase compared to control cells.*

exhibited a relatively low response to TRAIL/Apo2L-induced apoptosis (38) and was responsive to HDACi. In fact, K562 cells are able to differentiate into erythroid type cells after VPA, and BT treatment (38).

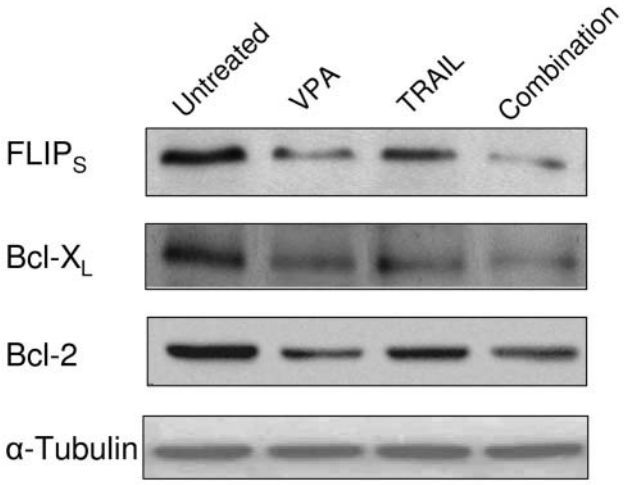


Figure 6. *Effects of VPA and TRAIL, alone and in sequential application on the expression of apoptosis-related protein. K562 cells were treated with 1 mM VPA for 24 hours, followed by 24 hours in the presence of 20 ng/mL TRAIL. Cell lysates were subjected to Western blotting as indicated.*

The ability of VPA to potentiate a TRAIL/Apo2L response in different cell types is not new. In a recent *ex vivo* work on lymphocytic leukemia cells (CLL) isolated from patients, VPA was shown to enhance the TRAIL/Apo2L response (57). Similarly, VPA reduced TRAIL/Apo2L-mediated cytotoxicity in cultured thoracic cancer cells through a mitochondria-dependent mechanism (58). Studies on cell lines confirmed the synergistic role of VPA when combined with TRAIL, as reported in hepatoma cells (59-60). What is less clear is how VPA exerts its apoptogenic activity. We firstly demonstrated that VPA acts on both the extrinsic and intrinsic apoptotic pathways. To our knowledge, this is the first report showing the down-regulation of Bcl-2 and Bcl-X_L, two antiapoptotic factors, following VPA treatment. In addition, the present study supports a pleiotropic activity of VPA on the apoptotic machinery. This observation contributes to explaining data from a previous report showing that SAHA, a pan-HDACi, was able to induce apoptosis in leukemia cells through both extrinsic and intrinsic death pathways (61).

The key result discussed in the present work regards the mode of action of VPA. We clearly showed, by means of different markers of the apoptotic process, that VPA acts as a pro-apoptotic factor, although it was not able to induce apoptosis *per se*. Increase of caspase-3 activity (Figure 4B and C), PARP-1 degradation (Figure 4D), down-regulation of FLIPs, Bcl-X_L and Bcl-2 (Figure 6) were all events observed after VPA treatment without TRAIL addition. However, these changes were not sufficient to induce cell death (Figure 4A). We hypothesize that the pleiotropic activity of VPA *prepares* cells for apoptosis, but is not

enough to overcome K562 apoptotic resistance. As an example, VPA down-regulated c-FLIPs (Figure 6), as also reported by others (60), but, unexpectedly, in our system no significant increase in caspase-8 expression and activation was observed; therefore, cell death was not detected. The main question as to how VPA enhances TRAIL/Apo2L-induced apoptosis is solved by data shown in Figure 5, where the ability of VPA to increase DR4 and DR5 expression was demonstrated. In summary, the scenario we imagine is the following: i) TRAIL/Apo2L-resistant cells, such as K562, do not respond to TRAIL/Apo2L due to their very low expression of TRAIL/Apo2L receptors, DR4 and DR5; ii) VPA exerts two main effects: it increases DR4 and DR5 expression, allowing TRAIL/Apo2L to bind to its receptors and activate the extrinsic apoptotic cascade, and increases cell sensitivity to apoptotic events by acting on both intrinsic (mitochondria) and extrinsic (DISC) pathways; iii) the combined effect of VPA plus TRAIL/Apo2L is now effective and synergistic, making cells prone to apoptosis. An indirect proof of this mechanism resides in the observation that the sequential treatment, VPA followed by TRAIL/Apo2L, was more effective than the co-incubation (Figure 4A).

Our data, in agreement with previous studies, supports the concept that triggering cell differentiation can modulate the cell death machinery, suggesting that cell differentiation may be employed in anticancer therapy by lowering the antiapoptotic barrier. As an example, K562 cells induced to differentiate, gain sensitivity to TRAIL/Apo2L by increased DR4 and DR5 expression (38), as also suggested by the present work. We confirmed in other cellular models that differentiation resulted in an increased expression of TRAIL/Apo2L receptors. In fact, qPCR analyses of HT29, U937, K562 and HL60 cell lines treated with BT, TPA (12-O-tetradecanoylphorbol-13-acetate), resveratrol, and TPA or DMSO (dimethyl sulfoxide), respectively, showed a constant and significant transcriptional increase of DR5 (data not shown). HDACi-enhanced Apo2L/TRAIL cytotoxicity is frequently attributed to increased surface expression of DR4 and/or DR5 (50-55). However, in CLL the most likely mechanism whereby HDACi sensitize cells to TRAIL/Apo2L appears to be located at the DISC level, with only a slight intervention of DR5 up-regulation (51). Moreover, in human multiple myeloma (MM) U266 cells, expressing significant levels of DR4 and caspase-8 but resistant to TRAIL/Apo2L, VPA causes the redistribution of DR4 to plasma membrane lipid rafts restoring sensitivity to DR4 signaling (62). It is noteworthy that VPA alone might be a promising candidate drug in the management of MM since it has been shown that the molecule has a time- and dose-dependent cytotoxic effect on the MM cells (63).

In increasing DR4 and DR5 expression, VPA may behave similarly to other HDACi, as reported in previous work, which induced hyper-acetylation of the genes promoter region of several members of the DR pathways including TRAIL/Apo2L, FAS ligand and FAS, through a mechanism engaging acetylation of the transcription factors SP1 or SP3, and consequent recruitment of CBP [CREB (cAMP response element-binding protein)-binding protein] (29). However, we cannot exclude that the increased expression of DR4 and DR5 (Figure 5) brought about VPA could also be due to mechanisms related to an increased stability of the DR4 and DR5 proteins.

To date, the clinical trials evaluating the therapeutic potential of TRAIL/Apo2L as anticancer agent are numerous. Drugs able to activate apoptosis through the death receptor pathway seems to be promising candidates for cancer therapy. However, resistance has seldom been reported in cancer cell towards TRAIL/Apo2L-induced apoptosis. The possibility of clinical use of the molecule may reside in TRAIL/Apo2L-based combination regimens to bypass resistance. The HDACi family might possess this attitude by virtue of its ability in reprogramming cell transcriptional profile and in inducing fundamental alterations in cell signaling pathways.

As suggested by this paper, VPA, for its already established pharmacokinetic, pharmacodynamic data and bioavailability, may represent an ideal candidate in a combined chemotherapeutic strategy, thus providing an attracting approach for the treatment of leukemias and other proliferative malignancies.

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