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ORIGINAL ARTICLE

Histone deacetylase inhibitors cooperate with IFN-γ to restore caspase-8 expression and overcome TRAIL resistance in cancers with silencing of caspase-8

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Evasion of apoptosis can be caused by epigenetic silencing of caspase-8, a key component of the extrinsic apoptosis pathway. Loss of caspase-8 correlates with poor prognosis in medulloblastoma, which highlights the relevance of strategies to upregulate caspase-8 to break apoptosis resistance. Here, we develop a new combinatorial approach, that is treatment using histone deacetylase inhibitors (HDACI) together with interferon (IFN)-γ, to restore caspase-8 expression and to overcome resistance to the death-receptor ligand TNF-related apoptosis-inducing ligand (TRAIL) in medulloblastoma *in vitro* and *in vivo*. HDACI, for example, valproic acid (VA), suberoylanilide hydroxamic acid (SAHA) and MS-275, cooperate with IFN-γ to upregulate caspase-8 in cancer cells lacking caspase-8, thereby restoring sensitivity to TRAIL-induced apoptosis. Molecular studies show that VA promotes histone acetylation and acts in concert with IFN-γ to stimulate caspase-8 promoter activity. The resulting increase in caspase-8 mRNA and protein expression leads to enhanced TRAIL-induced activation of caspase-8 at the death-inducing signaling complex, mitochondrial outer-membrane permeabilization and caspase-dependent cell death. Intriguingly, pharmacological or genetic inhibition of caspase-8 also abolishes the VA/IFN-γ-mediated sensitization for TRAIL-induced apoptosis. It is important to note that VA and IFN-γ restore caspase-8 expression and sensitivity to TRAIL in primary medulloblastoma samples and significantly potentiate TRAIL-mediated suppression of medulloblastoma growth *in vivo*. These findings provide the rationale for further (pre)clinical evaluation of VA and IFN-γ to restore caspase-8 expression and apoptosis sensitivity in cancers with caspase-8 silencing and open new perspectives to overcome TRAIL resistance.
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Introduction

Programmed cell death (apoptosis) is the cell's intrinsic death program that has a crucial role in the maintenance of tissue homeostasis (Hengartner, 2000). Evasion of apoptosis can contribute to tumor formation and treatment resistance, as the response of cancer cells to current treatment regimens is mediated by cell death in response to cytotoxic stimuli (Fulda and Debatin, 2006c).

Apoptosis signals through two principal pathways, the death-receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway (Fulda and Debatin, 2006c). Stimulation of death receptors, such as TNF-related apoptosis-inducing ligand (TRAIL) receptors results in activation of caspase-8 at the death-inducing signaling complex (DISC) (Ashkenazi, 2008). In the mitochondrial pathway, cytochrome *c* and Smac/direct IAP-binding protein with low pI (DIABLO) are released into the cytosol, which leads to caspase-3 activation through the cytochrome *c*/Apaf-1/caspase-9-containing apoptosome complex (Adams and Cory, 2007; Kroemer *et al.*, 2007). Mitochondrial contribution to death-receptor signaling is of special relevance in certain cell types, for example type-II cells, which depend on the release of mitochondrial factors for the full activation of caspase-3 and apoptosis (Scaffidi *et al.*, 1998; Fulda *et al.*, 2002; Ozoren and El-Deiry, 2002). The essential function of caspase-8 in death receptor-induced cell death has been shown in various *in vitro* and *in vivo* models, including caspase-8 knockout mice (Varfolomeev *et al.*, 1998).

Apoptosis resistance may be caused by loss or inactivation of key components of the apoptotic machinery (Fulda and Debatin, 2006b). For example, caspase-8 has been found to be frequently silenced by epigenetic mechanisms in several cancers (Grotzer *et al.*, 2000; Teitz *et al.*, 2000; Fulda *et al.*, 2001; Hopkins-Donaldson *et al.*, 2003; Fulda, 2008). It is important to note that loss of caspase-8 expression correlates with poor survival outcome in medulloblastoma (Pingoud-Meier *et al.*, 2003), indicating that caspase-8 is a relevant molecular target for the design of new treatment approaches. Previously, several agents have been

described to upregulate caspase-8 expression in cancers with epigenetically silenced caspase-8, including demethylating drugs, interferon (IFN)-γ, retinoic acid or the combination of IFN-γ and demethylating drugs, which in turn rendered cells susceptible to death-receptor- and also to drug-induced apoptosis (Grotzer *et al.*, 2000; Ruiz-Ruiz *et al.*, 2000; Fulda *et al.*, 2001, 2002; Fulda and Debatin, 2002, 2006a; Pingoud-Meier *et al.*, 2003; Yang *et al.*, 2003; Merchant *et al.*, 2004; Tekautz *et al.*, 2006; Lissat *et al.*, 2007; Meister *et al.*, 2007; Jiang *et al.*, 2008). TRAIL-receptor agonists are considered as promising cancer therapeutics and are currently under evaluation in early clinical trials (Ashkenazi and Herbst, 2008). However, loss of caspase-8 limits the use of TRAIL-receptor agonists in medulloblastoma, thus highlighting the need for effective approaches to restore caspase-8 levels (Fulda, 2008).

Remodeling of chromatin is a mechanism for regulating gene expression and involves reversible post-translational modification of amino acids in histone tails, in particular their acetylation status, which controls the accessibility of transcription factors to DNA (Nightingale *et al.*, 2006). Histone acetylation is governed by two groups of enzymes, that is histone acetyltransferases and histone deacetylases, and is frequently disturbed in human cancers (Roth *et al.*, 2001; Thiagalingam *et al.*, 2003). To target aberrant histone deacetylase activity, a variety of histone deacetylase inhibitors (HDACI) have been developed (Bolden *et al.*, 2006). It is postulated that HDACI cause accumulation of acetylated histones, which favors an open state of the chromatin, thereby facilitating transcription (Bolden *et al.*, 2006). Furthermore, HDACI can stimulate transcriptional activation through acetylation of non-histone proteins, including transcription factors (Bolden *et al.*, 2006). Searching for new mechanisms that regulate caspase-8 expression, we investigated whether histone deacetylation is involved in epigenetic silencing of caspase-8 in this study.

Results

HDACI and IFN-γ cooperate to restore caspase-8 expression

Searching for new strategies to restore caspase-8 expression in cancers with inactivation of caspase-8, we explored whether chromatin remodeling is involved in epigenetic silencing of caspase-8 expression. To address this question, we analyzed the effect of HDACI on histone acetylation and caspase-8 protein expression using western blotting in medulloblastoma as a model. We tested different classes of HDACI, including short-chain fatty acids, for example valproic acid (VA); hydroxymates, for example suberoylanilide hydroxamic acid (SAHA); benzamides, for example MS-275 and the experimental compound RO4474861 at a range of sub-toxic concentrations (Figure 1a and data not shown). Treatment with HDACI caused re-expression of caspase-8 in several medulloblastoma cell lines with silenced caspase-8, although to a variable degree depending on the inhibitor and the cell line (Figure 1a). Higher concentrations of HDACI were cytotoxic without upregulating caspase-8 (data not shown). IFN-γ was used as a positive control for upregulation of caspase-8 (Fulda *et al.*, 2002) (Figure 1a). HDACI-mediated acetylation of histone H4 or histone H3 did not directly correlate with re-expression of caspase-8 (Figure 1a), indicating that acetylation of non-histone proteins may also be involved in the HDACI-mediated upregulation of caspase-8.

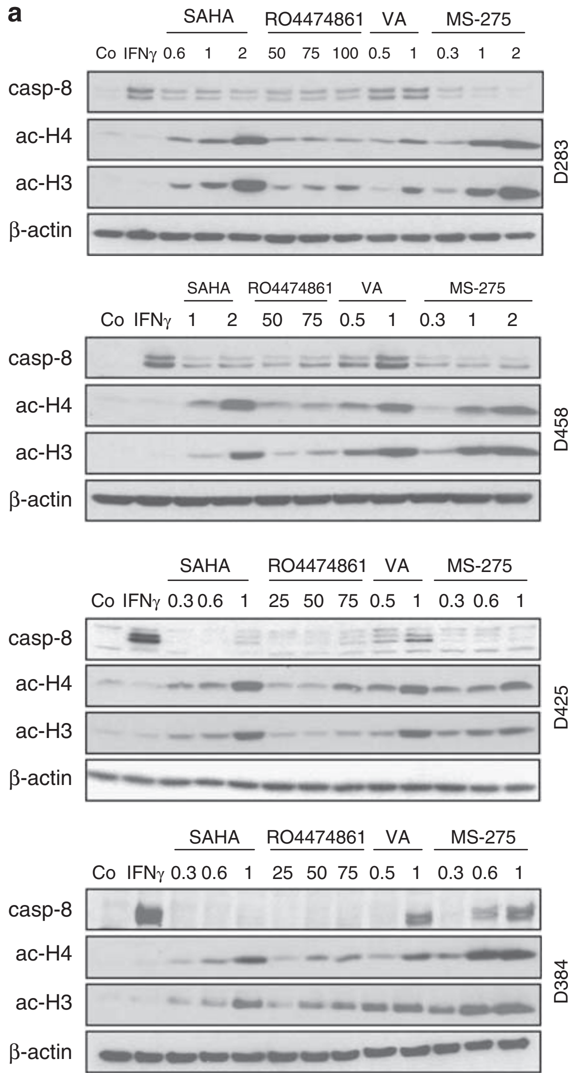
Moreover, we tested combinations of HDACI together with IFN-γ, as we previously identified a cooperative upregulation of caspase-8 by the combinatorial use of IFN-γ and the demethylation agent 5-Aza-2′-deoxycytidine (Fulda and Debatin, 2006a). For these experiments, we used the HDACI VA, as it consistently caused re-expression of caspase-8 in different medulloblastoma cell lines and as it is an established anti-epileptic drug, which is frequently part of an anticonvulsant drug regimen in patients with medulloblastoma and also under investigation for the treatment of medulloblastoma (Gottlicher *et al.*, 2001; Blaheta and Cinatl, 2002; Li *et al.*, 2005; Shu *et al.*, 2006). It is interesting to note that VA acted in concert with IFN-γ to stimulate caspase-8 expression in a dose- and time-dependent manner (Figure 1b and data not shown). To exclude the possibility that this cooperative interaction was restricted to one particular HDACI, we also tested additional HDACI in combination with IFN-γ. Similarly, MS-275 and SAHA cooperated with IFN-γ to enhance caspase-8 expression (Supplementary Figure 1a). To investigate whether the continuous presence of VA and IFN-γ was required to maintain caspase-8 expression, cells were incubated for 2 days with VA and IFN-γ and then placed into a drug-free medium. Caspase-8 levels declined over the next few days on the removal of VA and IFN-γ (data not shown), indicating that constant exposure to VA and IFN-γ was necessary to

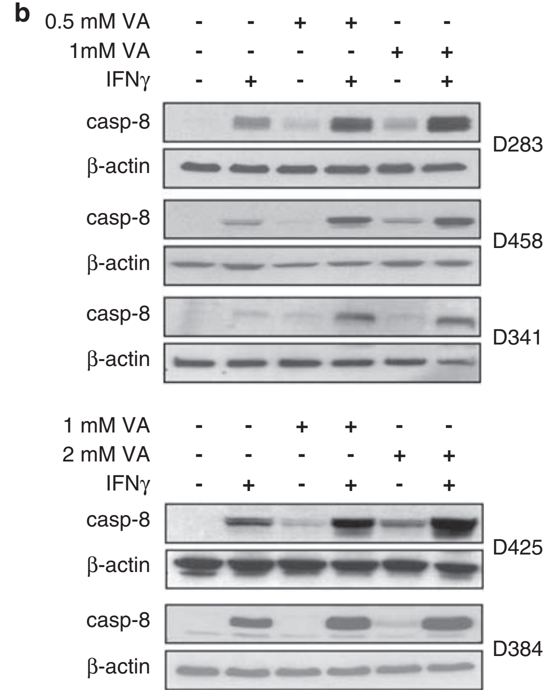
Figure 1 Histone deacetylase inhibitor (HDACI) and interferon (IFN)-γ cooperate to restore caspase-8 expression. (**a**) Cells were treated for 24 h with suberoylanilide hydroxamic acid (SAHA) (μM), RO4474861 (nM), valproic acid (VA) (mM), MS-275 (μM) or 1000 U/ml IFN-γ. Expression of caspase-8, acetylated histone H4, acetylated histone H3 and β-actin was assessed by western blotting. (**b**) Cells were treated for 48 h with VA and/or 1000 U/ml IFN-γ. Expression of caspase-8 and β-actin was assessed by western blotting. (**c**) Cells were treated for 12 h (D283) or 24 h (D458) with 1 mM (D283) or 0.5 mM (D458) VA and/or 1000 U/ml IFN-γ. Caspase-8 promoter activity was assessed by dual luciferase assay. Mean + s.e.m. of three independent experiments carried out in triplicate are shown; $*P<0.05$. (**d**) Cells were treated for 24 h with 1 mM (D283) or 0.5 mM (D458) VA and/or 1000 U/ml IFN-γ. Caspase-8 mRNA expression was assessed by real-time PCR analysis. A representative experiment of three independent experiments is shown.

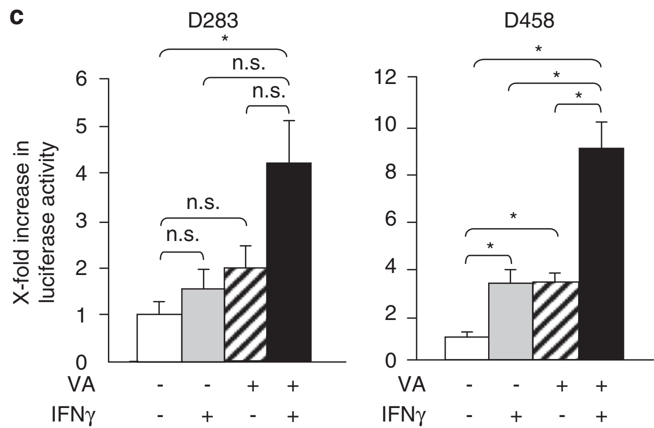
maintain caspase-8 levels. Together, this set of experiments shows that VA cooperates with IFN-γ to restore caspase-8 expression in medulloblastoma cells with loss of caspase-8.

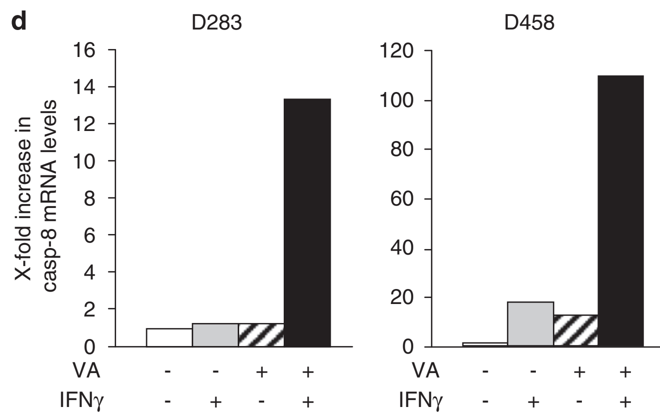
VA and IFN-γ cooperate to transcriptionally activate caspase-8 expression

Next, we wished to elucidate the molecular mechanisms underlying the cooperative interaction of VA and









IFN-γ to re-express caspase-8. First, analysis of the histone acetylation status showed that the addition of IFN-γ to VA did not substantially alter the VA-mediated acetylation of histone H4 (Supplementary Figure 1b). To determine whether the combination therapy stimulates caspase-8 promoter activity, we cloned the caspase-8 promoter into a promoterless luciferase construct and carried out luciferase promoter assays. The combined use of VA and IFN-γ was significantly more effective than either agent to trigger caspase-8 promoter activity (Figure 1c). Further, VA and IFN-γ acted in concert to increase caspase-8 mRNA levels (Figure 1d). Together, these experiments show that VA and IFN-γ cooperate to re-activate caspase-8 expression at the level of transcription.

VA and IFN-γ cooperate to enhance TRAIL-induced apoptosis and suppression of clonogenic growth

We then asked whether restoration of caspase-8 expression by VA and IFN-γ is functionally relevant and determines the responsiveness of medulloblastoma cells to TRAIL-induced apoptosis. To address this point, we pre-treated cells with VA and IFN-γ to upregulate caspase-8 levels and then added TRAIL to trigger apoptosis. Importantly, pre-treatment with the combination of VA and IFN-γ reversed the resistance to TRAIL and significantly enhanced TRAIL-mediated apoptosis (Figure 2a and data not shown). By comparison, no sensitization for TRAIL-induced apoptosis was found when VA and IFN-γ were administered concomitantly with TRAIL (data not shown), showing that pre-treatment with VA and IFN-γ to upregulate caspase-8 was required to render cells susceptible to TRAIL. Similarly, pre-exposure to VA and IFN-γ enhanced apoptosis induced by other death-receptor agonists, for example agonistic antibodies to TRAIL receptor 1, TRAIL receptor 2 or CD95 (data not shown and Supplementary Figure 1c).

Moreover, we carried out colony assays to assess the effect on long-term survival. Importantly, the combination of VA and IFN-γ significantly enhanced TRAIL-mediated suppression of clonogenic growth, whereas exposure to IFN-γ or VA alone did not significantly alter the anti-tumor activity of TRAIL (Figure 2b). Together, these findings show that VA and IFN-γ cooperate to render medulloblastoma cells sensitive to TRAIL-mediated apoptosis and also act in concert to potentiate TRAIL-induced suppression of clonogenic survival.

VA and IFN-γ cooperate to enhance TRAIL-induced activation of the caspase cascade

To understand how VA and IFN-γ act in concert to promote TRAIL-induced apoptosis, we systematically explored the effect of VA and IFN-γ on the TRAIL signaling pathway. First, we examined whether VA and IFN-γ alter surface expression of TRAIL receptors. Flow cytometric analysis showed no increase in surface expression of agonistic TRAIL receptors TRAIL-R1 and TRAIL-R2 or downregulation of antagonistic TRAIL receptors TRAIL-R3 and TRAIL-R4 upon exposure to VA, IFN-γ or the combination (data not shown). This indicates that alterations in surface expression of TRAIL receptors are probably not responsible for the observed increased sensitivity to TRAIL.

Next, we investigated DISC formation upon stimulation with TRAIL. Combined pre-treatment with VA and IFN-γ enhanced TRAIL-induced recruitment of caspase-8 into the DISC and cleavage of caspase-8 into the active p18 fragment at the DISC (Figure 3a). This was accompanied by a massive increase of the p18 active cleavage fragment of caspase-8 in whole-cell extracts, which was detected already 30 min after addition of TRAIL (Figure 3b). Furthermore, cleavage of caspase-3 into p17/12 active fragments, cleavage of caspase-9 into p37/35 fragments and cleavage of Bid was substantially increased in TRAIL-treated cells that were pre-incubated with the combination of VA/IFN-γ (Figure 3b). To examine whether the increased cleavage of caspases also translates into enhanced enzymatic caspase activity, we carried out caspase-activity assays. Similarly, pre-incubation with the combination of VA and IFN-γ significantly increased TRAIL-induced activation of caspase-8 and -3 (Figure 3c). Addition of the broad-range caspase inhibitor zVAD.fmk almost completely inhibited TRAIL-induced apoptosis (Figure 3d), indicating that apoptosis occurred in a caspase-dependent manner.

VA and IFN-γ cooperate to enhance TRAIL-induced mitochondrial damage

Moreover, we explored the involvement of the mitochondrial pathway in the VA/IFN-γ-mediated sensitization to TRAIL. Pre-treatment with VA and IFN-γ enhanced TRAIL-induced activation of Bax as indicated by an increase in Bax conformational change (Figure 4a). Analysis of mitochondrial alterations showed that the combined pre-treatment with VA/IFN-γ was more effective than either agent alone to sensitize cells for TRAIL-induced loss of mitochondrial membrane potential and cytochrome *c* release (Figures 4b and c). To test whether activation of the mitochondrial pathway is required for apoptosis induction, we blocked the mitochondrial pathway by overexpression of Bcl-2. Importantly, overexpression of Bcl-2 significantly reduced TRAIL-induced apoptosis in cells that were pre-treated with VA and IFN-γ, whereas it did not prevent caspase-8 upregulation upon exposure to VA and IFN-γ (Figure 4d). This shows that VA/IFN-γ-induced sensitization for TRAIL-triggered apoptosis depends on mitochondrial outer-membrane permeabilization, which is consistent with a type-II organization of the TRAIL pathway.

Requirement of caspase-8 for sensitization to TRAIL-induced apoptosis by VA and IFN-γ

As chromatin-modifying agents, such as HDACI, may change expression levels of various genes besides caspase-8, we further tested the specific contribution of caspase-8 using several independent approaches. In a first approach, a survey of pro- and anti-apoptotic proteins showed no consistent changes on combined treatment with VA and IFN-γ across the different cell

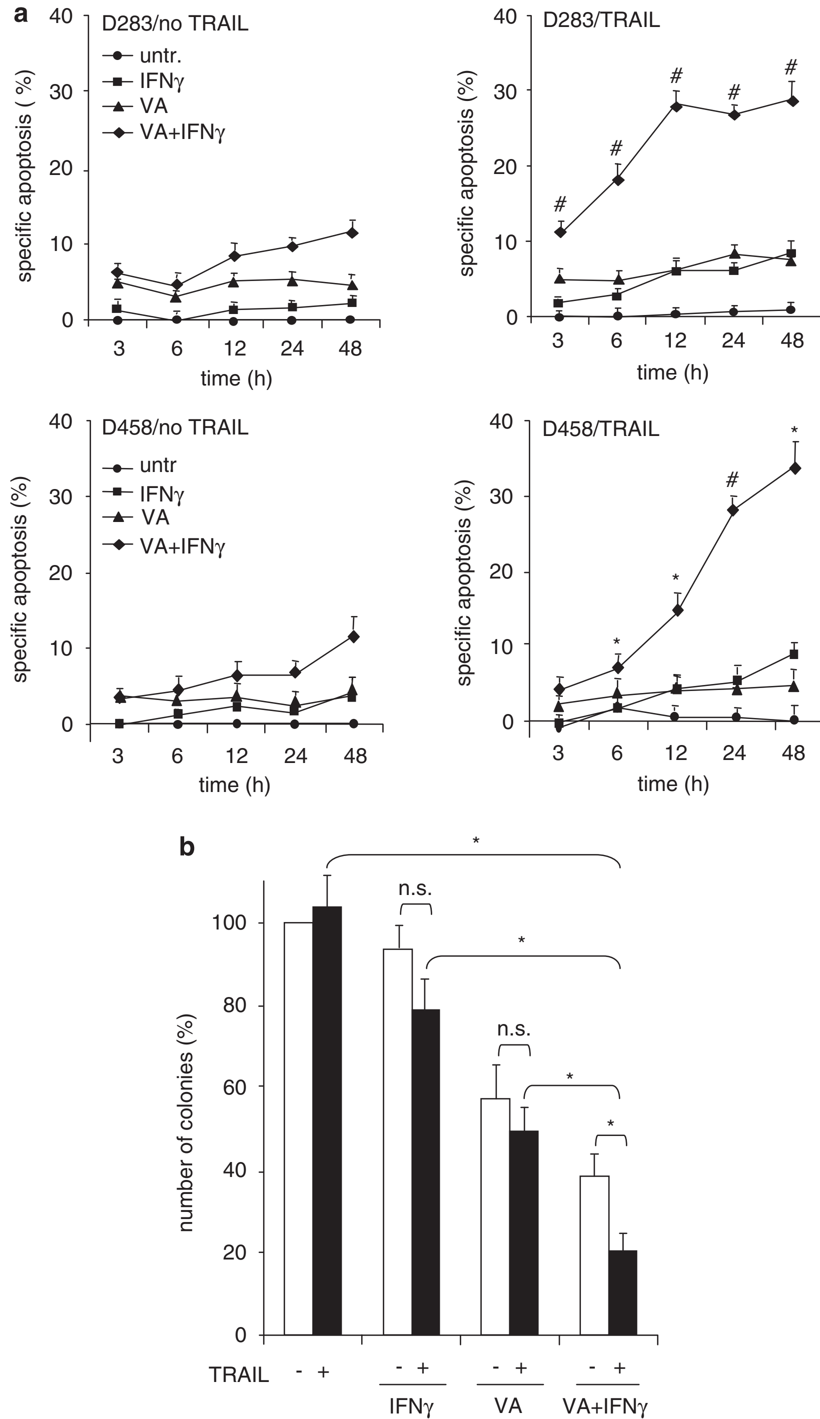


Figure 2 Valproic acid (VA) and interferon (IFN)-γ cooperate to enhance TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis and suppression of clonogenic growth. (**a**) Cells were treated for 48 h (D283) or 24 h (D458) with 1 mM (D283) or 0.5 mM (D458) VA and/or 1000 U/ml IFN-γ before 50 ng/ml TRAIL was added at the indicated time points. Apoptosis was determined as described in Materials and methods. (**b**) D283 cells were treated for 48 h with 1 mM VA and/or 1000 U/ml IFN-γ before 50 ng/ml TRAIL was added for 24 h. Colony formation was assessed as described in Materials and methods. In **a** and **b**, mean + s.e.m. of three independent experiments carried out in triplicate are shown; $*P<0.05$; $^{\#}P<0.001$.

lines examined compared with the strong upregulation of caspase-8 (Figures 1b, 5a and data not shown). Increased caspase-10 or decreased $FLIP_L$ or PED levels upon treatment with VA and IFN-γ were not consistently observed in all cell lines (Figure 5a and data not shown), indicating that these changes are probably not the primary mechanism of sensitization.

In a second approach, we specifically inhibited caspase-8 both pharmacologically and genetically. Administration of a pharmacological inhibitor of caspase-8 prevented the

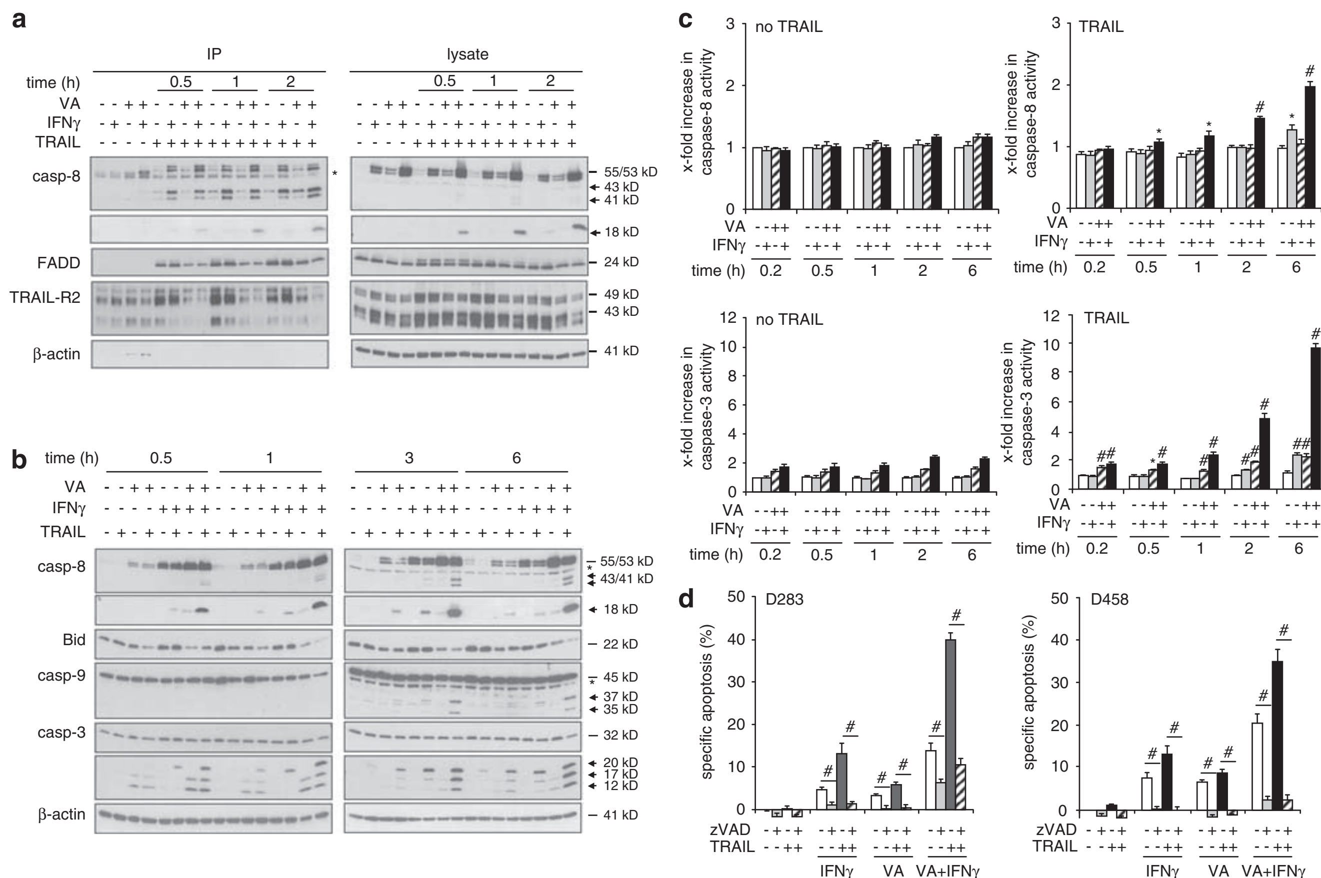


Figure 3 Valproic acid (VA) and interferon (IFN)-γ cooperate to enhance TNF-related apoptosis-inducing ligand (TRAIL)-induced activation of caspases. D283 cells were treated for 48 h with 1 mM VA and/or 1000 U/ml IFN-γ before Flag-tagged TRAIL was added at the indicated times (**a**) or 50 ng/ml TRAIL was added at the indicated times (**b**, **c**) or for 48 h (**d**). The TRAIL DISC was analyzed by immunoprecipitation as described in Materials and methods (**a**), activation of caspases and Bid was analyzed by western blotting (**b**, arrows indicate cleavage fragments), caspase activity was determined by enzymatic caspase assay (**c**), apoptosis was determined in the presence or absence of 50 μM zVAD.fmk as described in Materials and methods (**d**). In **a** and **b**, a representative experiment of three independent experiments is shown. In **c** and **d**, mean + s.e.m. of three independent experiments carried out in triplicate are shown; *$P<0.05$; #$P<0.001$. Asterisks indicate unspecific bands.

sensitization for TRAIL-induced apoptosis in cells, in which caspase-8 was upregulated by VA and IFN-γ (Figure 5b). Even more noteworthy is that preventing the VA/IFN-γ-mediated upregulation of caspase-8 by RNA interference also completely blocked apoptosis, which was induced by the addition of TRAIL (Figures 5c and d). Together, these findings show that inhibition of caspase-8 function also inhibits the VA/IFN-γ-mediated sensitization for TRAIL-induced apoptosis. Thus, caspase-8 is a crucial mediator of the VA and IFN-γ-mediated sensitization for TRAIL.

VA and IFN-γ cooperate to restore caspase-8 expression and sensitivity to TRAIL in neuroblastoma cells and in primary medulloblastoma cells without toxicity on normal PBLs

To exclude the possibility that our findings are restricted to medulloblastoma, we extended our studies to neuroblastoma as another prototypic cancer with epigenetic silencing of caspase-8 (Teitz *et al*., 2000; Fulda, 2008). Similarly, VA and IFN-γ cooperated to upregulate caspase-8 expression and to enhance TRAIL-induced apoptosis in neuroblastoma cells (Figure 6a).

Moreover, we validated the results that we obtained in cell lines also in primary medulloblastoma samples from surgical specimens. Caspase-8 was undetectable in primary specimens consistent with previous reports that caspase-8 is frequently silenced in medulloblastoma (Grotzer *et al*., 2000; Pingoud-Meier *et al*., 2003). VA and IFN-γ acted in concert to upregulate caspase-8 even in primary medulloblastoma samples (Figure 6b, left panel). Intriguingly, pre-treatment with VA and IFN-γ primed cells for TRAIL-induced apoptosis in the sample, in which caspase-8 was markedly upregulated (Figure 6b). These experiments provide proof-of-principle that VA and IFN-γ cooperate to upregulate caspase-8 expression and to prime patient-derived medulloblastoma cells for TRAIL-induced apoptosis.

We also extended our studies to normal peripheral blood lymphocytes (PBLs) from healthy donors

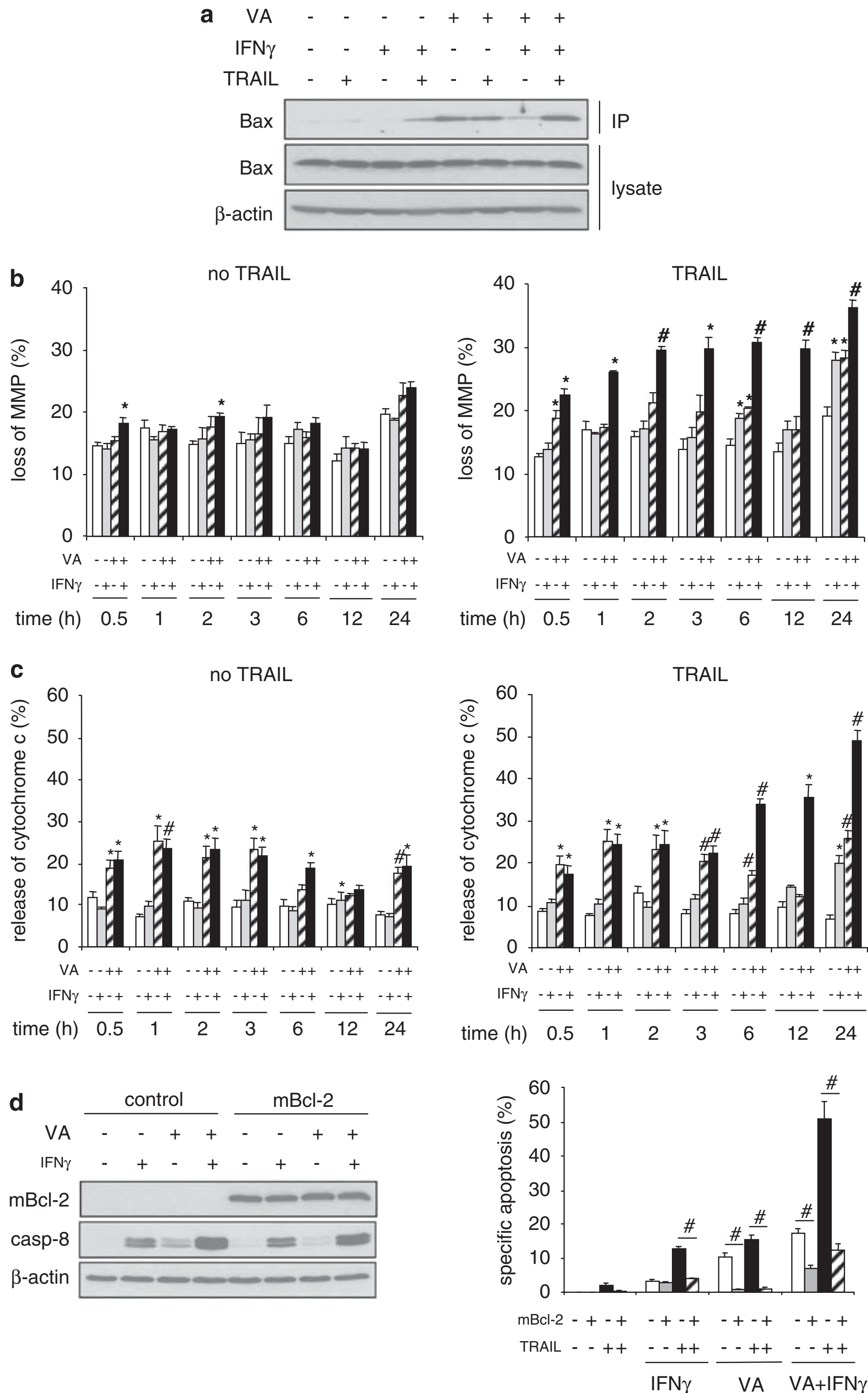


Figure 4 Valproic acid (VA) and interferon (IFN)-γ cooperate to enhance TNF-related apoptosis-inducing ligand (TRAIL)-induced mitochondrial damage. (**a**–**c**), D283 cells were treated for 48 h with 1 mM VA and/or 1000 U/ml IFN-γ before 50 ng/ml TRAIL was added for 3 h (**a**) or at the indicated times (**b**, **c**). In (**a**), Bax conformational change was determined by immunoprecipitation (IP), Bax expression in lysates served as controls. Mitochondrial transmembrane potential (**b**) and cytochrome *c* release (**c**) were assessed by FACS analysis. (**d**) D283 cells transduced with mouse Bcl-2 (mBcl-2) or empty vector were treated for 48 h with 1 mM VA and/or 1000 U/ml IFN-γ. Expression of caspase-8, mBcl-2 and β-actin was assessed by western blotting (**d**, left panel). Apoptosis after addition of 50 ng/ml TRAIL for 48 h was determined as described in Materials and methods (**d**, right panel). In **a** and left panel of **d**, a representative experiment of three independent experiments is shown. In **b**, **c** and right panel of **d**, mean + s.e.m. of three independent experiments carried out in triplicate are shown; $*P<0.05$; $^{\#}P<0.001$.

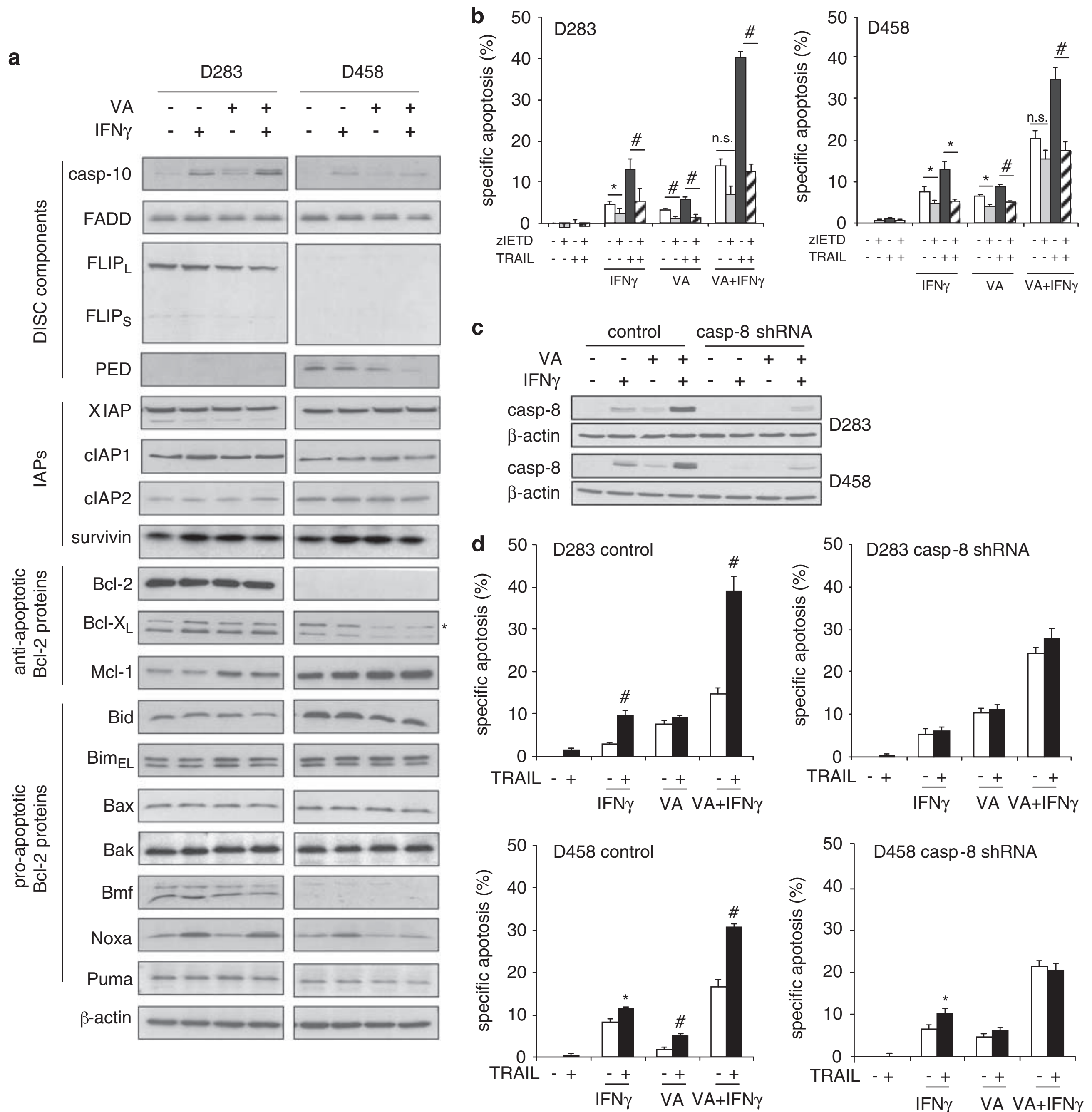


Figure 5 Requirement of caspase-8 for the sensitization to TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by valproic acid (VA) and interferon (IFN)-γ. (**a**) Cells were treated for 48 h with 1 mM (D283) or 0.5 mM (D458) VA and/or 1000 U/ml IFN-γ. Expression of apoptosis-regulatory proteins was determined by western blotting. (**b**) D283 cells were treated for 48 h with 1 mM VA and/or 1000 U/ml IFN-γ before 50 ng/ml TRAIL was added for 48 h in the presence or absence of 50 μM zIETD.fmk. Apoptosis was determined as described in Materials and methods. (**c**, **d**) Cells transduced with a vector containing caspase-8 shRNA or control vector were treated for 48 h (D283) or 24 h (D458) with 1 mM (D283) or 0.5 mM (D458) VA and/or 1000 U/ml IFN-γ. In (**c**), expression of caspase-8 and β-actin was assessed by western blotting. In (**d**), apoptosis after addition of 50 ng/ml TRAIL for 48 h (D283) or 24 h (D458) was determined as described in Materials and methods. In **a** and **c**, a representative experiment of three independent experiments is shown. In **b** and **d**, mean + s.e.m. of three independent experiments carried out in triplicate are shown; $^{*}P<0.05$; $^{\#}P<0.001$.

to investigate whether the combination treatment is cytotoxic to non-malignant cells. Treatment using VA and IFN-γ did not alter caspase-8 levels or the response towards TRAIL in unstimulated or stimulated PBLs (Supplementary Figure 2). We assayed both unstimulated PBLs as well as activated PBLs 6 days after

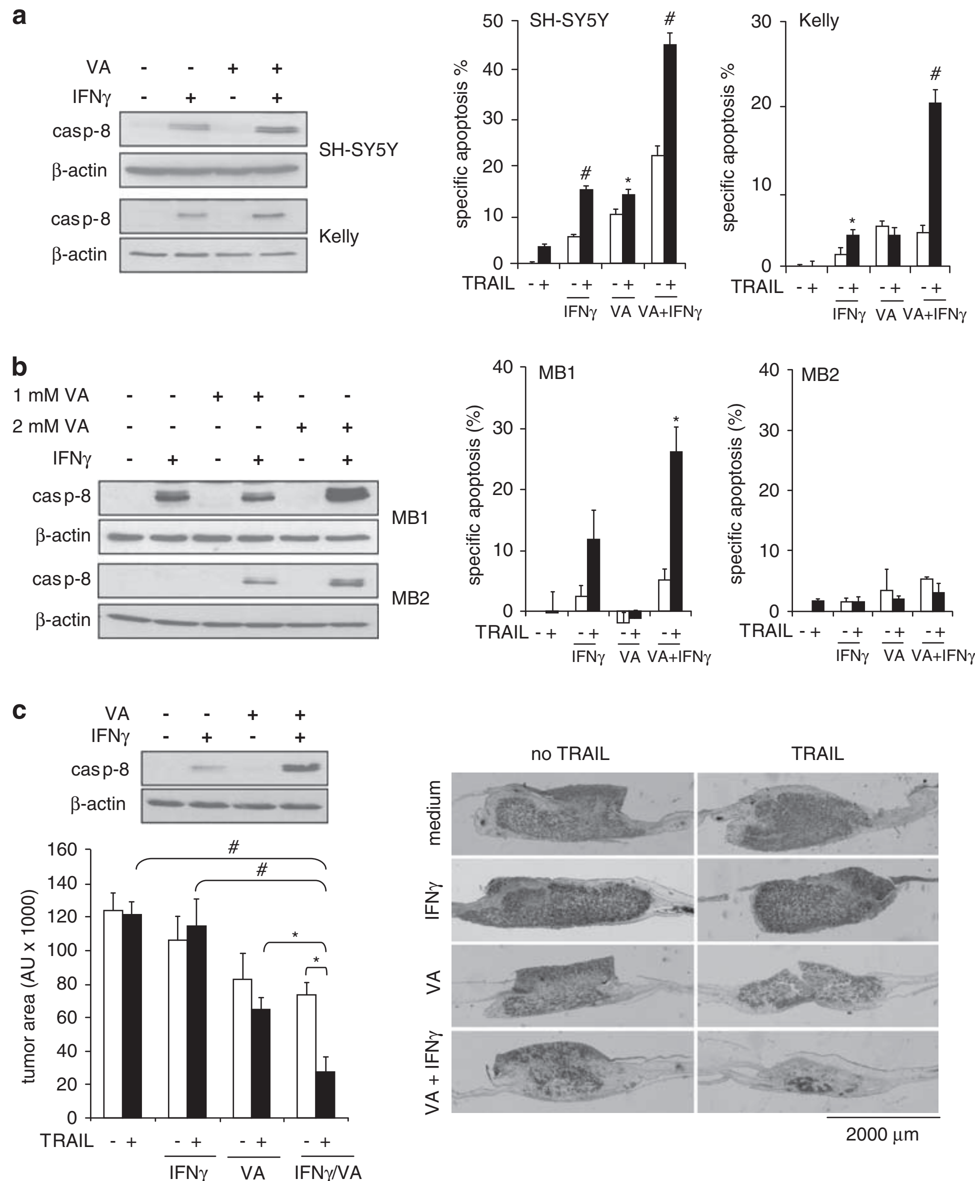


Figure 6 Valproic acid (VA) and interferon (IFN)-γ cooperate to restore caspase-8 expression and TNF-related apoptosis-inducing ligand (TRAIL) sensitivity in neuroblastoma cells, primary medulloblastoma cells and *in vivo*. (**a**) Neuroblastoma cells were treated for 48 h with 2 mM VA and/or 100 U/ml IFN-γ (SH-SY5Y) or 0.5 mM VA and/or 10 U/ml IFN-γ (Kelly). Expression of caspase-8 and β-actin was assessed by western blotting (left panel). Apoptosis after addition of 50 ng/ml TRAIL for 24 h was determined as described in Materials and methods (middle and right panels). (**b**) Primary medulloblastoma cells were treated with 1 or 2 mM VA and/or 1000 U/ml IFN-γ for 48 h. Expression of caspase-8 and β-actin was assessed by western blotting (left panel). Apoptosis after treatment with 2 mM VA and/or 1000 U/ml IFN-γ for 48 h (MB1) or 24 h (MB2) followed by the addition of 50 ng/ml TRAIL for 24 h was determined as described in Materials and methods (middle and right panels). In (**a**), a representative blot of three independent experiments is shown, bars represent mean + s.e.m. of three independent experiments carried out in triplicate; in (**b**), data from one experiment are shown, bars represent mean + s.e.m. of triplicate. *$P<0.05$; #$P<0.001$. (**c**) D283 cells were treated for 48 h with 1 mM VA and/or 1000 U/ml IFN-γ before seeded on the chorioallantoic membrane (CAM) of chicken embryos and treated with TRAIL. Tumor growth was analyzed using hematoxylin and eosin-stained paraffin sections of the CAM. Expression of caspase-8 and β-actin after treatment with VA and/or IFN-γ for 48 h was assessed by western blotting (**c**, upper left panel). Tumor area and representative pictures of hematoxylin and eosin-stained sections of the CAM are shown (**c**, lower left panel and right panel; bars: 2000 μm). Error bars indicate mean + s.e.m. of eight samples per group; *$P<0.05$; #$P<0.001$. Similar results were obtained in three independent experiments.

stimulation with phytohemagglutinin, as sensitivity of peripheral lymphocytes to CD95-mediated apoptosis has been reported to increase on mitogen stimulation (Peter *et al.*, 1997; Pingoud-Meier *et al.*, 2003) and this stimulus was thus used as a positive control (Supplementary Figure 2b). This set of experiments shows that VA and IFN-γ cooperate to restore caspase-8 expression and sensitivity to TRAIL in different cancers with loss of caspase-8 as well as in primary medulloblastoma cells without reversing the lack of toxicity of TRAIL on normal PBLs, pointing to some tumor specificity.

VA and IFN-γ cooperate with TRAIL to suppress medulloblastoma growth in vivo

Finally, we evaluated the anti-tumor activity of our approach *in vivo* using the chorioallantoic membrane (CAM) model, an established *in vivo* tumor model that has also been used to study the role of caspase-8 in neuroblastoma (Kuefer *et al*., 2004; Stupack *et al*., 2006; Vogler *et al*., 2008, 2009). To this end, medulloblastoma cells were pre-treated with VA, IFN-γ or the combination to upregulate caspase-8, seeded on the CAM of chicken embryos, allowed to form tumors and treated for 3 days with TRAIL in the presence or absence of VA and/or IFN-γ. Intriguingly, pre-treatment using VA and IFN-γ, which was most effective in restoring caspase-8 expression, also significantly enhanced the TRAIL-induced suppression of medulloblastoma growth *in vivo* (Figure 6c). This shows that the combination of VA and IFN-γ renders medulloblastoma cells sensitive to TRAIL-induced suppression of tumor growth *in vivo*.

Discussion

Loss of caspase-8 has recently been reported to correlate with poor prognosis in patients with medulloblastoma (Pingoud-Meier *et al*., 2003). Silencing of caspase-8, a key signaling molecule in the death-receptor pathway, also precludes the successful application of TRAIL-receptor agonists in medulloblastoma which represent promising apoptosis-based cancer therapeutics (Ashkenazi and Herbst, 2008). Hence, new strategies are required to target caspase-8 inactivation in medulloblastoma to overcome TRAIL resistance.

New combination strategy of HDACI and IFN-γ to restore caspase-8 expression and TRAIL sensitivity

Here, we identify histone deacetylation as a new mechanism of epigenetic inactivation of caspase-8. We develop a new combinatorial approach to restore caspase-8 expression and sensitivity to TRAIL in medulloblastoma *in vitro* and *in vivo* using the well-established anti-convulsant VA, which was recently identified as HDACI (Gottlicher *et al*., 2001; Blaheta and Cinatl, 2002), in combination with IFN-γ. Our study unravels a previously unknown cooperative interaction between VA and IFN-γ to transcriptionally activate caspase-8 expression, thereby restoring TRAIL sensitivity in cancer cells that lack caspase-8. Analysis of the underlying molecular mechanisms of the synergistic interaction shows that VA and IFN-γ cooperate at the transcriptional level to re-activate caspase-8 expression. Hyperacetylation of histones upon exposure to VA, which coincides with caspase-8 upregulation, indicates that VA may cause a more open chromatin structure that facilitates transcription of caspase-8. This idea is supported by the increase in caspase-8 promoter activity upon combined treatment with VA and IFN-γ compared with either agent alone. However, acetylation of non-histone proteins may also contribute to the upregulation of caspase-8, as we found no strict correlation between the acetylation of histones and caspase-8 expression upon treatment with different HDACI. The cooperative effect of VA and IFN-γ on the caspase-8 promoter results in increased caspase-8 mRNA and protein levels, which in turn leads to enhanced activation of caspase-8 at the DISC upon TRAIL stimulation and thus an amplification of the entire TRAIL signaling cascade. Accordingly, re-expression of caspase-8 by pre-treatment with VA and IFN-γ enhances cleavage of caspase-8 into active fragments, caspase-8 enzymatic activity, Bid cleavage, Bax activation, mitochondrial outer membrane permeabilization, caspase-3 activation and caspase-dependent apoptotic cell death. It is important to note that an intact mitochondrial signaling pathway turned out to be required for TRAIL-induced cell death in medulloblastoma cells, as Bcl-2 overexpression blocked TRAIL-induced apoptosis, consistent with a type-II organization of the TRAIL signaling pathway in medulloblastoma cells. Importantly, combination treatment using VA and IFN-γ switches TRAIL-resistant medulloblastoma cells to TRAIL responders even in long-term assays, in primary patient-derived medulloblastoma cells and in an *in vivo* model, indicating that it is a powerful strategy to overcome TRAIL resistance in cancers with loss of caspase-8.

Several independent pieces of evidence underline the specific contribution of caspase-8 for the VA/IFN-γ-mediated sensitization for TRAIL. First, pre-treatment rather than concomitant administration of VA and IFN-γ is necessary to confer sensitivity for TRAIL, indicating that modulation of gene expression, for example upregulation of caspase-8, may be required before the administration of TRAIL. Further, a survey of pro- and anti-apoptotic molecules shows that caspase-8 is the candidate that is massively upregulated by the combination of VA and IFN-γ. More specifically, pharmacological inhibition of caspase-8 also abolishes the sensitization for TRAIL-induced apoptosis that is provided by VA and IFN-γ. Intriguingly, preventing the VA/IFN-γ-mediated upregulation of caspase-8 using RNA interference also abrogates the switch into a TRAIL-sensitive phenotype. Together, these findings provide convincing evidence that caspase-8 is a key mediator of VA/IFN-γ-mediated sensitization towards TRAIL. This does not exclude the possibility that additional factors may contribute to the observed sensitization to TRAIL, as HDACI and IFN-γ can affect expression levels of a panel of target genes and may also influence signal-transduction pathways at the post-transcriptional level (Van Boxel-Dezaire and Stark, 2007).

Several agents have been tested in recent years for their potential to upregulate caspase-8 in cancers with caspase-8 silencing, such as medulloblastoma. For example, treatment with IFN-γ resulted in upregulation of caspase-8 and enhanced the sensitivity of medulloblastoma cells to TRAIL and also to radio- and chemotherapy in short-term *in vitro* assays (Grotzer *et al*., 2000; Ruiz-Ruiz *et al*., 2000; Fulda *et al*., 2001, 2002; Fulda and Debatin, 2002, 2006a; Pingoud-Meier

et al., 2003; Yang *et al*., 2003; Tekautz *et al*., 2006; Lissat *et al*., 2007; Meister *et al*., 2007; Jiang *et al*., 2008). In addition, re-expression of caspase-8 has been reported for demethylation agents, such as 5-Aza-2′-deoxycytidine (Grotzer *et al*., 2000; Teitz *et al*., 2000; Fulda *et al*., 2001), retinoid acid (Jiang *et al*., 2008) and the combination of IFN-γ and 5-Aza-2′-deoxycytidine (Fulda and Debatin, 2006a). Although there is currently much interest to exploit HDACI for cancer therapy (Bolden *et al*., 2006), they have not yet been tested in the context of caspase-8-negative medulloblastoma. Thus, the novelty of our study particularly resides in the demonstration that the combination of VA with IFN-γ presents a new strategy, which is more effective than either agent alone in restoring caspase-8 expression and responsiveness to TRAIL in medulloblastoma cell lines, and most importantly, also in primary medulloblastoma samples and in an *in vivo* model.

Implications for apoptosis-based cancer therapies

Our study has several important implications for the development of biology-based targeted therapies for caspase-8-deficient cancers, such as medulloblastoma to overcome some forms of apoptosis resistance. First, caspase-8 represents a clinically relevant molecular target in medulloblastoma, as loss of caspase-8 has previously been identified as an indicator of poor prognosis in this cancer (Pingoud-Meier *et al*., 2003). To this end, epigenetic silencing of caspase-8 was reported to correlate with resistance to TRAIL-induced apoptosis in medulloblastoma cell lines and with unfavorable survival outcome of children with medulloblastoma in a clinical study (Grotzer *et al*., 2000; Pingoud-Meier *et al*., 2003).

Second, it is feasible that our strategy to use VA and IFN-γ for re-expression of caspase-8 can be translated into clinical application, as both VA and IFN-γ are drugs approved by the Food and Drug Administration, which have also already been tested in pediatric oncology. IFN-γ has previously been used in an immunotherapy trial in children with neuroblastoma and has been shown to be well tolerated in the treatment of chronic granulumatous disease and atopic dermatitis (Wexler *et al*., 1992; Jonasch and Haluska, 2001). As VA is frequently part of an anti-epileptic regimen in patients with medulloblastoma (Guerrini, 2006) and has been evaluated in a clinical trial in children with brain tumors (for further information visit http://www.clinicaltrials.gov), the feasibility is high to translate it into medical application in an alternative setting, that is in TRAIL-based combination protocols. Thus, the rational use of established drugs in a new context, that is VA together with IFN-γ to restore caspase-8 expression followed by the administration of TRAIL to trigger the death-receptor pathway of apoptosis, constitutes an innovative strategy to bypass TRAIL resistance in cancers, such as medulloblastoma.

Third, restoration of TRAIL sensitivity by re-expression of caspase-8 may open new perspectives for the use of TRAIL-receptor agonists in cancers with silencing of caspase-8. Recombinant soluble TRAIL and agonistic TRAIL-receptor antibodies provide clinically applicable tools to directly trigger programmed cell death in tumors and are currently evaluated in early clinical trials (Ashkenazi and Herbst, 2008). However, primary resistance to TRAIL in cancers with loss of caspase-8 limits the clinical application of TRAIL-receptors agonists (Grotzer *et al*., 2000; Fulda *et al*., 2001). By showing that restoration of caspase-8 expression can switch medulloblastoma cells from non-responders to TRAIL responders using either soluble TRAIL or agonistic TRAIL-receptor antibodies, our findings indicate that pre-treatment with VA and IFN-γ presents an effective approach to restore sensitivity to TRAIL.

Fourth, restoration of a functional death-receptor pathway upon re-expression of caspase-8 is also expected to contribute to effective tumor elimination through the innate and acquired immune system, as TRAIL and CD95 ligand are effector molecules of the innate immune response and cytotoxic T cells, respectively (Krammer, 2000; Smyth *et al*., 2003).

Finally, biology-based new treatment approaches are probably critical for improving the prognosis of medulloblastoma patients, as conventional therapies and risk stratification have already been optimized in recent years (Rutkowski *et al*., 2007). In conclusion, by showing that combined treatment with VA and IFN-γ restores caspase-8 expression and primes medulloblastoma for TRAIL-induced apoptosis *in vitro* and *in vivo*, our findings open new perspectives to overcome TRAIL resistance in cancers with epigenetic silencing of caspase-8.

Materials and methods

Cell culture and reagents

Cell lines were cultured as described by Fulda and Debatin (2006a) using IMEMZO, minimum essential medium, RPMI1640 or Dulbecco's modified eagle medium (Life Technologies Inc., Eggenstein, Germany). The experiments using medulloblastoma tumor samples were approved by the local Ethics Committee. TRAIL was from R&D Systems Inc. (Wiesbaden, Germany), IFN-γ from Biochrom (Berlin, Germany) and all chemicals from Sigma (Deisenhofen, Germany) unless indicated otherwise.

Transduction

The shRNA sequence targeting caspase-8 5′-gggtcatgctctatc agat-3′ (Wagner *et al*., 2004) was cloned into pRETRO-SUPER as described by Vogler *et al*. (2007). Stable bulk cultures were generated by selection with 1 μg/ml puromycin. For Bcl-2 overexpression, cells were transduced with pMSCV vector containing mouse Bcl-2 or empty vector using the packaging cell line PT67 (BD Biosciences, Heidelberg, Germany). Stable cell lines were selected using 10 μg/ml blasticidin (Invitrogen).

Western blot analysis

Western blot analysis was carried out as described by Fulda *et al*. (1997) using the following antibodies: caspase-8

(ApoTech Corporation, Epalinges, Switzerland); caspase-3 and Bim (Cell Signaling, Beverly, MA, USA); caspase-9, caspase-2, XIAP, Bcl-2, Apaf-1, Bak, Bcl-X_L, FADD and p21 (BD Biosciences); caspase-10 (MoBiTec, Göttingen, Germany); Bcl-2 (Zymed, South San Francisco, CA, USA); survivin, cIAP1 and Bid (R&D Systems Inc.); cIAP2 (Epitomics, Burlingame, CA, USA); acetylated histone H4 (Calbiochem, San Diego, CA, USA); acetylated histone H3 and Bax NT (Upstate Biotechnology, Lake Placid, NY, USA); Bmf, FLIP and Noxa (Alexis, Grünberg, Germany); Mcl-1 (Stressgen, Ann Arbor, MI, USA); PED (kindly provided by F Beguinot, Naples, Italy); Puma and β-actin (Sigma) and goat-anti-mouse IgG, goat-anti-rabbit IgG and donkey-anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or goat-anti-rat IgG (Dianova, Hamburg, Germany) conjugated to horseradish peroxidase. Enhanced chemiluminescence was used for detection (Amersham Bioscience, Freiburg, Germany).

Bax immunoprecipitation
Cells were lysed in CHAPS lysis buffer (10 mM HEPES (pH 7.4); 150 mM NaCl; 1% CHAPS). A total of 2 mg protein was incubated with 16 μg mouse anti-Bax antibody (6A7, Sigma) overnight at 4 °C followed by addition of 10 μl Dynabeads Pan Mouse IgG (Dako, Hamburg, Germany), incubated for 2 h at 4 °C, washed with CHAPS lysis buffer and were analyzed by western blotting using BaxNT antibody (Upstate Biotechnology).

Determination of apoptosis, colony formation, mitochondrial membrane potential, cytochrome c *release and caspase activity*
Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei as described by Vogler *et al*. (2007). For clonogenic assay, cells were seeded in a 1% methylcellulose medium (StemCell Technologies, Vancouver, Canada) and colonies with more than 20 cells per colony were counted after 8–10 days. Mitochondrial membrane potential, cytochrome *c* release, caspase activity and DISC immunoprecipitation were determined as described by Mohr *et al*. (2004); Giagkousiklidis *et al*. (2005).

Reverse transcription and PCR analysis
RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany). A total of 1 μg RNA was transcribed using ImPro-II Reverse transcription system (Promega, Madison, WI, USA). TaqMan Universal PCR Master Mix and TaqMan gene-expression assay for caspase-8 (plate I.D. 299238) from Applied Biosystems (Foster City, CA, USA) were used for real-time PCR analysis according to the manufacturer's instructions.

Cloning and luciferase assays
A 1200-bp fragment containing the human caspase-8 promoter (Banelli *et al*., 2002) was cloned from pBL-CAT3 (kindly provided by M Romani, Genova, Italy) into pBluescript KS (+) using restriction enzymes *Hind*III and *Xho*I. The 1.2-kb fragment was then cloned using restriction enzymes *Xba*I and *Xho*I into the promoterless pGL3 luciferase reporter vector (Promega, Madison, WI, USA) by cohesive attachment of *Nhe*I and *Xba*I. Correct insertion was verified by sequencing. The Dual-Luciferase Reporter Assay System (Promega) was used to determine firefly and renilla luciferase activities according to the manufacturer's instructions.

CAM assay
CAM assay was done as described previously (Kuefer *et al*., 2004; Vogler *et al*., 2008). Briefly, cells were pre-treated for 48 h with VA and/or IFN-γ, and 3×10^6 cells were implanted on fertilized chicken eggs on day 8 of incubation and were treated with 1 mg VA and/or 0.31 μg IFN-γ (Biochrom) and 35 ng TRAIL for 4 days, sampled with the surrounding CAM, fixed in 4% paraformaldehyde, paraffin embedded, cut in 5 μm sections and were histological analyzed using 1:1 hematoxyline and 0.5% eosin. Images were digitally recorded at a magnification of $\times 2$ with an AX70 microscope (Olympus, Center Valley, PA, USA), tumor areas were analyzed with SimplePCI digital imaging software (Compix Inc., Cranberry, PA, USA).

Statistical analysis
The statistical significance was assessed using Student's *t*-test or Mann–Whitney *U* test, where appropriate, using Winstat (R. Fitch Software, Bad Krozingen, Germany) or SPSS (SPSS GmbH Software, Munich, Germany) software.

Conflict of Interest

The authors declare no conflict of interest.

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