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# Differential immunotoxicity of histone deacetylase inhibitors on malignant and naïve hepatocytes

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**Keywords:** TNF, TRAIL, CD95L, cFLIP, XIAP, hepatocellular carcinoma, adverse side effect, primary hepatocytes

## Footnotes

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**Abbreviation:** ActD, Actinomycin D; CBHA, m-Carboxycinnamic acid bis-hydroxamide; HDAC, Histone deacetylase; HDI, Histone deacetylase inhibitor; M344, N-Hydroxy-7-(4-dimethylaminobenzol)aminoheptanamide; VPA, Valproic acid

## Abstract

Histone deacetylases (HD) represent a novel target in cancer treatment, in particular of scattered small tumours such as the hepatocellular carcinoma (HCC). However, only few studies address the toxicological impact of HD Inhibitors (HDIs) on malignantly transformed cells versus primary hepatocytes. We examined whether and how different classes of HDIs sensitise the human HCC cell line HepG2, primary healthy murine and human liver cells towards the death receptor agonists TNF $\alpha$  and CD95L.

Apicidin, M344 (N-hydroxy-7-(4-dimethylaminobenzol)aminoheptanamide), CBHA (m-carboxycinnamic acid bis-hydroxamide) and VPA (valproic acid) sensitised liver cell cultures towards CD95-triggered apoptosis with the following potency: Apicidin > M344  $\approx$  CBHA >> VPA. Apicidin sensitised towards CD95 also in the intact organ, i.e. in the isolated perfused mouse liver. No significant sensitisation towards TNF $\alpha$  was found in vitro.

Western blot analysis showed that all HDIs studied downregulated the anti-apoptotic protein cFLIP, but only VPA additionally affected the expression level of XIAP. Furthermore, in models of the intrinsic apoptosis pathway, i.e. in HepG2 cells treated with Melphalan and in primary hepatocytes irradiated with UV light, only VPA exhibited significant sensitisation.

These findings extend the biochemical, pharmacological and toxicological basis for HDI therapy and provide a caveat for clinical use in patients with an accompanying critical inflammatory state in which the CD95 system might be pre-activated.

## Introduction

Pharmacological manipulation of epigenetic mechanisms by the chemically heterogeneous group of histone deacetylase inhibitors (HDI) provides a novel approach for cancer therapy. Their ability to induce growth arrest, differentiation and apoptosis in cell lines derived from different malignant tumours has been extensively investigated in the last three decades (Candido et al. 1978). In contrast, there are only a few studies which address the immunotoxicological action of HDIs on primary, healthy cells.

Class I and class II HDACs are zinc-dependent enzymes for which a number of structurally diverse natural and synthetic inhibitors have been identified (Johnstone, 2002). Compounds that are shown to inhibit HDAC activity by binding to the zinc-containing catalytic domains comprise six structurally distinct classes: small molecular weight carboxylates (e.g. valproic acid), hydroxamic acids (e.g. CBHA), benzamides (e.g. MS-275), epoxyketones (e.g. trapoxin), cyclic tetrapeptides (e.g. Apicidin), and hybrid molecules (e.g. cyclic hydroxamic acid-containing peptide (CHAP 31)) (Finnin et al. 1999; Vannini et al. 2004; Drummond et al. 2005). The physiological effect of HDIs is based primarily on modification of the equilibrium of HDACs and histone acetyl transferases in favour of the latter ones, resulting in opened chromatin and eventually in upregulation as well as repression of different genes (Melnick and Licht 2002; Peart et al. 2005).

One important pharmacological application of HDIs is cancer treatment as mono- or combinatorial therapy (Faraco et al. 2006; Rasheed et al. 2007; Sailhamer et al. 2008). The currently accepted rationale for their anti-tumour efficacy is suppression of tumour cell proliferation, metastasis, angiogenesis, invasion and survival with little or no toxicity to normal cells. Thus, inhibition of histone deacetylases – resulting in upregulation of pro-apoptotic proteins together with a downregulation of anti-apoptotic proteins – potentially represents an efficient tool to sensitise otherwise largely chemoresistant cancer cells towards cell death.

One of the most important systems of cell death induction is the tumour necrosis factor receptor (TNFR) gene superfamily, including TNFR-1 and Fas/CD95 (Ashkenazi 2002). Pathophysiologically, the TNFR system is also involved in a wide range of hepatic diseases such as viral or alcoholic hepatitis, Wilson's disease and graft versus host disease (Chu et al. 1995; Galle et al. 1995; Luo et al. 1997). Binding of the complementary death activator results in death receptor trimerisation, which allows binding of adapter molecules such as FADD or TRADD, thus forming the death-inducing signalling complex (DISC) and activating the caspase cascade which is responsible for most morphological features of

apoptotically dying cells (Ashkenazi and Dixit 1999; Peter and Krammer 2003). Inhibition of initiator and/ or executioner caspases by antiapoptotic factors like cFLIP or XIAP is therefore an efficient cellular mechanism to suppress apoptosis signalling (Bortner and Cidlowski 2002; Irmeler et al. 1997; Nagata 1997; Kataoka et al. 1998).

Due to its special and outstanding role of detoxification, viral clearance and primary filter for circulating tumor cells within the organism, the liver is exposed to a large number of toxic metabolites, reactive oxygen species and pathogens and hence predisposed for malign transformation and for metastases from other organs (Koren et al. 1992). It is therefore not surprising that HCC is one of the most frequent cancers worldwide with increasing incidence. There are good reasons to assume that systemically administered HDIs affect also healthy cells. Hence, this study focuses on HDI-mediated alterations in the pathway of death receptor-dependent apoptosis in transformed and healthy hepatocytes. In order to differentiate between singularity and general rule, we examined a broad spectrum of HDI classes which serve as lead structures for the clinical development. Here, we provide evidence that targeted manipulation of the balance of gene expression on the epigenetic level will also influence the physiology of normal, healthy cells. These results offer a basis for an extended understanding of HDIs and may flag potential risks in the development and clinical use of HDI-based anti-cancer drugs.

## Materials and Methods

**Substances.** Activating anti-CD95 antibody (Jo2) and polyclonal IgG horseradish peroxidase-coupled secondary antibody (goat anti-mouse) were from PharMingen (San Diego, CA, USA), CD95L from Dr. Elisa Ferrando-May, Konstanz, recombinant mouse TNF $\alpha$  from Innogenetics (Ghent, Belgium). Mouse monoclonal anti-XIAP was purchased from BD Biosciences Pharmingen (Heidelberg, Germany) and anti-cFLIP was delivered by Santa Cruz Biotechnology (Heidelberg, Germany). The rabbit polyclonal anti- $\beta$ -Actin was from New England Biolabs (Frankfurt am Main, Germany). Sodium valproate (Ergenyl<sup>®</sup>) was purchased from Sanofi-Synthelabo (Berlin, Germany); Apicidin from Sigma-Aldrich (Taufkirchen, Germany), M344 and CBHA from Calbiochem (Schwalbach, Germany). SAHA, LAQ-824 and MS-275 were kindly provided from Altana Pharma AG, Konstanz. The caspase substrate Ac-DEVD-afc was obtained from Biomol (Hamburg, Germany). AlamarBlue<sup>™</sup> was from BioSource (Solingen, Germany). The cytotoxicity detection kit (LDH) was from Roche

Diagnostics GmbH (Mannheim, Germany). The Bradford reagent was purchased from Bio-Rad Laboratories (Munich, Germany). Other chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany).

**Animals.** C57Bl6 wild type mice (25 g) were raised in the animal breeding facility of the University of Konstanz and were held at 22°C and 55% humidity with a day/night cycle of 12 h under adherence to NIH and the European Council (directive 86/609/EEC) guidelines.

**Isolation and culture of mouse hepatocytes.** Hepatocytes were isolated from 8 weeks old mice according to Seglen (Seglen 1973) and cultured as described (Leist, Gantner et al. 1994). To separate hepatocytes, the obtained cell suspension was resuspended in 32 ml Hanks' solution (HBSS), mixed with 20 ml Percoll solution and centrifuged at  $800 \times g$  for 10 min. Hepatocytes were plated in 200  $\mu$ l RPMI 1640 medium containing 10% FCS, 100  $\mu$ g/ml penicillin and 100 U/ml streptomycin in collagen-coated 24-well plates at a density of  $8 \times 10^4$  hepatocytes per well. Adherence was allowed for 3 h before the medium exchange to RPMI 1640 without FCS. Incubations were performed at 37°C, 5% CO<sub>2</sub>, 40% O<sub>2</sub> and 55% N<sub>2</sub>.

**Culture of primary human hepatocytes.** Primary human hepatocytes isolated from pathologically inconspicuous specimens were kindly provided from Dr. A. Nuessler (Charité, Berlin). Cells were cultured in RPMI 1640 supplemented with 10% FCS in 24-well plates and maintained at 5% CO<sub>2</sub>, 37°C.

**Treatment of cells.** Two hours before starting experiments, the culture medium of primary human or murine hepatocytes was exchanged to RPMI 1640 without FCS. The cells were preincubated with HDIs 3 hours before treatment with cytokines or Melphalan, respectively. DMSO alone was used as control. To carry out UV-radiation, the supernatant was removed, control cells were covered and the hepatocytes were irradiated. After achievement of the energy dose, medium and corresponding stimuli were immediately added.

**Isolated liver perfusion.** After *i.v.* injection of 150 mg/kg pentobarbital-sodium and 0.8 mg/kg heparin, the vena portae and the vena cava inferior were cannulated and ligated, and the liver was perfused until blood-free. Perfusion was carried out with 147 mM NaCl; 5.36 mM KCl, 0.34 mM Na<sub>2</sub>HPO<sub>4</sub>; 0.44 mM KH<sub>2</sub>PO<sub>4</sub>; 0.77 mM MgSO<sub>4</sub>; 10 mM D-Glucose; 9

mM HEPES; pH: 7.3; 325-330 mOsm with a total recirculating volume of 25 ml under constant pressure of 21.4 mm Hg and an osmolality of 330 mOsm. Temperature was 37°C and oxygenation with pure oxygen was done at 500 mbar. Technical equipment was from Hugo Sachs Electronics (March-Hugstetten, Germany). The extent of liver damage was assessed by measuring alanine aminotransferase (ALT) activity with an EPOS 5060 analyzer (Netheler & Hinz, Hamburg, Germany) according to the method of Bergmeyer [Bergmeyer HU et al., 1978] and calculated as U/l plasma.

**Cytotoxicity assay.** The tetrazolium dye AlamarBlue™ assay was used. The signal of solvent-treated cells was set to 0% of cells lysed with 0.1% Triton® X-100 to 100% cytotoxicity. The release lactate dehydrogenase (LDH) was determined using the Cytotoxicity Detection Kit in culture supernatants (S), and in the remaining cell monolayer (C) after lysis with 0.1% Triton® X-100. The percentage of LDH release was calculated as the ratio S/(S+C).

**Caspase-3/-7 activity assay.** Cells were washed three times with phosphate-buffered saline (PBS) and lysed with hypotonic extraction buffer (25 mM HEPES, pH 7.5, 5 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM PEFA-block plus 0.1% Triton® X-100). After centrifugation (15 min, 13,000 x g, 4°C) supernatants were frozen at -80°C. 7-amino-4-trifluoromethylcoumarin (afc) formation was followed in 50 mM HEPES, pH 7.4, 1% sucrose, 0.1% CHAPS, 10 mM DTT, 50 μM fluorogenic substrate DEVD-afc (N-acetyl-asp-glu-val-asp-afc, Biomol, Hamburg, Germany) for 30 min at 37°C. The fluorometer plate Victor<sup>2</sup> (Wallac Instruments, Turku, Finland) was set at 385 nm excitation and 505 nm emission wavelength. Protein was determined with the Pierce-Assay (Pierce, Rockford, IL, USA). Specific caspase-3/-7-like protease activity was calculated in pmol free afc per min (μU) and mg protein using serially diluted standards (0-5 μM afc).

**Preparation of S100 Fraction.** HepG2 cells (6 x 10<sup>6</sup>) were washed with ice-cold PBS and lysed with extraction buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1mM EGTA, 1 mM DTT). After 15 min on ice, cells were passed 15 times through a G27 needle, extracts were centrifuged at 900 x g (10 min, 4°C) and finally at 50,000 x g for 1 h. The S100 fraction was frozen in liquid nitrogen and stored at -80°C.

**Induction of caspase-3-like activity in a cell-free reconstitution system *via* intrinsic pathway.** 35  $\mu$ l of S100 fraction ( $\sim 3 \mu\text{g}/\mu\text{l}$ ), 5  $\mu\text{M}$  cytochrome c and 1 mM dATP were occasionally mixed and incubated at 37°C for 30 min. Caspase-3/-7 activation was determined in 10  $\mu$ l aliquots.

**Transient transfection.** According to TransPass<sup>TM</sup> D2 from New England Biolabs (Frankfurt am Main, Germany) HepG2 cells were transfected in 6-well with the human caspase-8 construct or the vector pCMV2 (Sigma-Aldrich, Taufkirchen, Germany) one day after plating. After four hours, transfection complexes were removed and incubation with HDIs was started after medium exchange to RPMI 1640 with FCS. After sixteen hours caspase-3/-7 activation was determined.

**Western Blot.** Cultured cells were lysed with lysis buffer (250 mM sucrose, 50 mM Tris-HCl, 5 mM imidazole, 2.5 mM EDTA, 2.5 mM DTT, 0.1% Triton<sup>®</sup> X-100, pH 7.40) and protein concentration was measured using the Bradford protein assay. Briefly, an aliquot of each sample equivalent to 40  $\mu\text{g}$  protein was boiled after addition of the appropriate amount of 5x sample buffer (5 mM EDTA, 162 mM DTT, 5% SDS, 50% glycerol, 0.51 bromophenol blue, 188 mM Tris, pH 8.8). The samples were separated on 10% SDS-polyacrylamide gels (PAGE) and electrophoretically transferred onto nitrocellulose filters using the Bio-Rad electrotransfer system (Bio-Rad Laboratories, Munich, Germany). Equal transfer was verified by Ponceau staining of the membranes. Antigen-antibody complexes were visualised with HRP-coupled secondary antibodies (goat anti-mouse and goat anti-rabbit, Dianova, Hamburg, Germany) and a custom-made ECL detection system (2.5 mM luminol, 0.4 mM para-coumaric acid, 10 mM Tris base, 0.15 l H<sub>2</sub>O<sub>2</sub>, pH 8.5).

**Statistics.** Data are means  $\pm$  SD. Statistics were calculated by one-way analysis of variance (ANOVA) followed by Dunnett's or Bonferroni's Multiple Comparison Test of controls *vs.* treated groups. All *vs.* all comparisons were done using Tukey Multiple Comparison Test. GraphPad Prism<sup>®</sup> 4.01 (GraphPad Software Inc.) was used and  $p < 0.05$  was considered significant.

## Results

To provide a comprehensive characterisation of the interaction of HDIs with death-inducing cytokines in the liver, HepG2 and cultured primary murine hepatocytes (pmH) were

incubated with increasing concentrations of the four structurally dissimilar HDIs Apicidin, CBHA, M344 or VPA in combination with TNF $\alpha$  or CD95L, respectively. Because murine cells are not sensitive towards TRAIL, only HepG2 cells were exposed to TRAIL. All four HDIs studied enhanced CD95L- and TRAIL-, but not TNF $\alpha$ - induced toxicity in HepG2 cells in a concentration-dependent manner. In primary murine liver cells, the HDIs studied also increased cytotoxicity towards CD95L, but not towards TNF $\alpha$  (Figure 1).

For further experiments, the concentration windows for the individual HDIs were defined by the optimal ratio of basal cytotoxicity *versus* enhancement of cytokine-induced toxicity. With this experimental set up, the previous results were confirmed: M344, CBHA, Apicidin and valproic acid caused a concentration-dependent, selective toxicity of CD95L in HepG2 and pmH. The numerically combined results in Table 1 (graphs not shown) display that equipotent concentrations for CD95L-induced cytotoxicity in HepG2 cells are 1  $\mu$ M M344, 5  $\mu$ M CBHA, 0.5  $\mu$ M Apicidin or 12.5 mM VPA. With the exception of VPA, equipotent concentrations were significantly higher in primary murine hepatocytes (Apicidin: 10x; M344: 100x; CBHA: 20x) as compared to the human hepatoma cell line HepG2.

In order to corroborate these findings in the whole organ we used isolated recirculating mouse liver perfusion. This organ model also comprises non-parenchymal cells in their physiological composition and environment. In contrast to control perfusion with saline – which did not result in any significant hepatotoxicity for up to 480 min as assessed by ALT release – the extent of cytotoxicity induced by  $\alpha$ CD95 was concentration-dependent (Fig 2, open symbols). In the presence of the HDI Apicidin, however, hepatotoxicity was significantly increased at any  $\alpha$ CD95 concentration studied (Figure 2, closed symbols). This finding demonstrates that the cell culture experiments translate to the whole organ, i.e. to healthy cells in their natural environment.

In order to further substantiate the relevance for humans, the influence of HDIs on CD95-induced apoptosis was examined in primary human hepatocyte cultures. These hepatocytes were isolated from pathological inconspicuous specimens obtained from patients undergoing hepatic resections for the therapy of hepatic tumours. For these crucial experiments, the spectrum of HDIs studied was extended by clinical candidates such as SAHA; MS-275 and LAQ824. As shown in Figure 3, any of the six HDIs caused a significant sensitisation towards CD95L-induced apoptosis in human liver cells. This finding demonstrates that the conditions observed in hepatic cell lines and animal tissue – *in vitro* as well as *in situ* – are likely to apply also for humans.

By recording the time course of caspase-3-like activation in relation to cytotoxicity in HDI-sensitised HepG2 and pmH over 24 hours, we wanted to gather information on the question of cause or consequence as to the activation of executioner caspases. Hepatocytes and hepatoma cells incubated with CD95L alone exhibited only low cytotoxicity and caspase activity, respectively, in comparison to combined treatment with HDIs. For any of the compounds tested, a peak in caspase activity was reached around 12 hours after cell death induction. Data are shown for HepG2 cells treated with the combination of CBHA and CD95L / TNF- $\alpha$  / TRAIL (Figure 4). We conclude that cytotoxicity is likely to be the consequence of caspase-activation, an interpretation which is confirmed by the finding that in the presence of 1.5  $\mu$ M of the pan-caspase inhibitor zVAD, CD95 triggered toxicity in sensitised cells was completely blunted (data not shown).

The transmission and execution of apoptosis signals is regulated by a network of accessory proteins. Since HDIs modulate gene expression, up- and/or downregulation of pro- and/or anti-apoptotic proteins represents a likely mechanism of sensitisation towards apoptosis. Based on previous information in non-hepatocyte cells, we analysed the expression levels of the anti-apoptotic factors cFLIP and XIAP by Western blot in cell lysates from HepG2 treated with 5  $\mu$ M CBHA and 12.5 mM VPA and in pmH treated with 100  $\mu$ M CBHA and 12.5 mM VPA for up to 21 hours (Figure 5). Indeed, in response to CBHA or VPA the protein level of cFLIP decreased from 3 to 6 hours, was barely detectable at 15 hours, and became essentially undetectable in both cell types. The expression of XIAP was also decreased over time in both HepG2 and pmH, but only in the presence of VPA.

Subsequent experiments examined whether HDI-mediated sensitisation is effected by the *extrinsic* or rather the *intrinsic* apoptosis signalling pathway. To this end, two cell-free models where S100 cytosolic lysates from HepG2 cells are incubated with HDIs were used. One set of experiments used lysates containing co-expressed recombinant caspase-8 (extrinsic pathway model), whereas dATP/cytochrome c was used in the other set (intrinsic pathway model). Activation and/or modulation of either pathway by HDIs was determined by assessing caspase-3/-7 like activity. As seen in Figure 6A, essentially no pan-caspase activity was detectable in the absence of caspase-8 expression. Compared to controls, a significant increase in caspase-3/-7 activity was detected in the extracts from HepG2 cells pre-treated with any of the indicated HDIs. In contrast to the inherent modulating action on the *extrinsic* pathway of all tested HDIs, a sensitising effect on the *intrinsic* pathway was exclusively observed for VPA (Figure 6B). The presence of the control agent PETCM, which antagonizes prothymosin- $\alpha$ , a negative regulator of apoptosome pathway, significantly enhanced the

caspase-3-like activity in this system. In contrast to CBHA, M344 and Apicidin, only VPA increased caspase-3/-7 activity in this experimental set up. As only VPA diminished expression of XIAP, i.e. a negative regulator of the intrinsic mitochondrial pathway of apoptosis, we tested the hypothesis that VPA also leads to a synergistic cytotoxic effect in combination with a DNA-damaging treatment. Indeed, combination of therapeutic concentrations of VPA and melphalan (in HepG2) and UV-radiation (in pmH), respectively, applied in concentrations that are non-toxic when used alone, caused a fulminant induction of cell death (Figure 7).

## Discussion

This study provides further insight into the molecular events that occur in receptor-dependent apoptosis in the presence of various classes of HDIs. It seems remarkable that such a chemically diverse class of compounds as the HDIs studied here share the common property to break resistance against apoptosis. Since such resistance phenomena often limit the therapeutic options of chemotherapeutics in solid tumours, the co-administration of HDIs and immunotherapeutic has been tested in preclinical and clinical investigations (Witzig et al. 2000; Hernandez et al. 2001). However, the problem of this approach is that the requirement of constant systemic exposure to any HDI (Rosato and Grant 2003) is associated with a transient accumulation of HDIs in the liver, representing a potential source of hepatotoxic side effects.

HCC is one of the most frequent cancers worldwide with increasing incidence and the liver is the target of many other cancers due to metastasis. Especially hepatocytes possess a high density of death-receptors, conjectively due to their vulnerable position towards hepatotropic viruses. This implies that any influence on the complex balance between pro-apoptotic and pro-survival signals is critical for the integrity and hence function of this vital organ.

The clinical incentives to use TRAIL in combination with HDIs to selectively eradicate HCC cells (Pathil et al. 2006; Dzieran et al., 2008) raise the question about possible adverse effects of TRAIL and – because of their tight evolutionary relationship – also CD95 on primary hepatocytes. In order to define conditions for rational combinatorial therapy regimens, it is of particular interest to study interference of HDAC inhibition with death receptor-induced cell death in healthy hepatocytes and to localize the site(s) of interference. To this end, we focused on a panel of four cell-permeable, chemically different HDIs with known differential effects in identical biological systems (Mitsiades et al. 2004; Peart et al. 2005). All of them overcame apoptotic resistance towards CD95L- and TRAIL-induced apoptosis in HepG2 cells. These

findings confirm previous work with other HDIs in diverse cell lines (Ashkenazi 2002; Chopin et al. 2002). Additional studies on the combination of HDIs with CD95L or TNF in nontransformed primary human and mouse hepatocytes revealed that HDIs are generally able to decrease the apoptotic threshold. From a purely experimental view it is an interesting fact that murine cells are TRAIL-resistant, meaning that an *in vivo* mouse approach is not possible. In order to account for the complexity and cellular heterogeneity of the liver, we used the isolated perfused mouse liver exposed to HDIs/CD95L as *in situ* model as the closest mimic of the *in vivo* situation. This exemplary model confirmed the combined cytotoxic activity of HDI and death receptor ligand.

It was not anticipated that in the presence of HDIs, the cytokine TNF $\alpha$  neither activates caspases nor induces cytotoxicity. This finding is different from data reported for the breast cancer cell lines MCF-7, T47-D and BT-20 (Chopin et al. 2004). It is another example for the assumption that alterations in the expression pattern, induced by the treatment with HDIs, are cell type-specific (Drummond et al. 2005), and that analogy conclusions should be avoided.

Another important point is that in contrast to CD95L or TRAIL, TNF $\alpha$  signalling is more complex, because here two different receptors activated pathways of both cell survival (e.g. via activation of NF $\kappa$ B or ERK signalling pathway) and cell death (Beg and Baltimore 1996; Wallach 1997). Since HDAC inhibition is known to induce transcriptional activation of NF $\kappa$ B by acetylation of the RelA/p65 subunit, a protection against TNF is likely (Dai, 2005).

The fact that HDIs sensitise malignant as well as nontransformed hepatocytes towards CD95L and TRAIL emphasizes the similarities of both signalling pathways. The sensitisation towards TRAIL is of considerable clinical interest as shown by several studies (Pathil, 2006; Dzieran, 2008). Ever more relevant, the sensitisation towards CD95L may give rise to serious adverse side effects, as this cytokine is active within various liver-related diseases and immunological states prevalent especially in the pathogenesis of HCC.

Our study attributes the sensitising action of HDIs to cell death to the extrinsic pathway of apoptosis, presumably by downregulation of the antiapoptotic protein cFLIP, which is known to be highly upregulated in resistant tumour cells and to block DISC assembly as well as caspase-8 recruitment (Irmeler et al. 1997; Yeh et al. 2000).

The particular properties of VPA also need to be considered, as VPA is a long-approved drug for the treatment of epilepsy and because only recently VPA was shown to reduce lung tumour growth of breast cancer cells (Goettlicher et al. 2001) and induce apoptosis in human melanoma cell lines (Facchetti et al. 2004). Of all tested HDIs, only VPA enhanced caspase-3 like activity induced by caspase-9 in the S100 cell free apoptosome model. Since XIAP in

addition to caspases -3 and -7 also binds the initiator caspase -9, this anti-apoptotic protein is a more potent inhibitor of the intrinsic than the extrinsic pathway (Bortner and Cidlowski 2002). Therefore, the downregulation of XIAP induced by VPA but not the other HDIs tested could provide an explanation for these findings. Also in the models of cytotoxicity induced by the DNA-alkylating agent Melphalan and UV-radiation, respectively, only VPA caused sensitisation *via* the intrinsic mitochondria-dependent pathway. This opens the perspective of breaking chemoresistance towards chemotherapy or radiation by HDIs affecting the mitochondrial way of apoptosis in combination with VPA.

In summary, the view that cytotoxicity of HDIs per se is restricted to fast dividing, malignantly transformed cells needs to be refined. We provide experimental evidence that sensitisation toward cytokine-induced cell death may also affect healthy cells, at least in the case of the CD95 system in the liver. In this organ, pre-activation especially the CD95 system e.g. in alcohol-induced hepatitis, Wilson's disease or Hepatitis B (which often end in HCC) represents a major caveat. Acknowledging the potential of HDIs, we would like to raise awareness for the toxic potential inherent to this class of drugs which inevitably pass the liver.

#### Disclosure statement

The author discloses no conflicts of interests

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**Table 1.** Equipotent HDI concentrations for CD95L-induced cytotoxicity in HepG2 and pmH. Based on the ratio of the basic cytotoxicity *versus* the enhancement in the presence of the ligands, constant concentrations of the HDIs, inducing identical rates of cell death in HepG2 and pmH, respectively, were defined and the sensitising effects were investigated with regard to the concentration dependencies of CD95L-induced cell death.

**Figure 1.** Concentration dependence of the modulation of receptor mediated cell death by HDIs in HepG2 cells (**left**) and pmH (**right**)

Cells were incubated without or with increasing concentrations of VPA (**A**), Apicidin (**B**), CBHA (**C**) and M344 (**D**). After preincubation for 3 hours, the cytokines TRAIL (100 ng/ml; diamond shaped), TNF $\alpha$  (100 ng/ml; triangle shaped) and CD95L (10% v/v; square) were added. HDIs alone were plotted as open circles. Cytotoxicity was determined after 18 hours by the AlamarBlue<sup>TM</sup> viability assay. Data represent means  $\pm$  SD.

**Figure 2:**  $\alpha$ CD95-mediated hepatotoxicity in isolated perfused mouse liver exposed to the HDI apicidin.

Isolated livers from C57B16 mice were perfused in situ with 5  $\mu$ M apicidin and different concentrations of  $\alpha$ CD95 as indicated up to 8 h. At various time points liver injury was quantified by measurement of the liver-specific enzyme alanine aminotransferase (ALT).

Data represent means  $\pm$  SD \*:  $p < 0.05$  for  $\alpha$ CD95 vs  $\alpha$ CD95 + apicidin. (Bonferroni's post test)

**Figure 3:** CD95L-triggered cytotoxicity in the presence of HDIs in primary human hepatocytes.

Primary human liver cells were preincubated with CBHA (100  $\mu$ M), VPA (12.5 mM), apicidin (5  $\mu$ M), SAHA (100  $\mu$ M), MS-275 (100  $\mu$ M) or LAQ824 (100  $\mu$ M) 3 hours before treatment with CD95L (10% v/v). After 18 h cytotoxicity was assessed by determination of LDH release.

Data represent means  $\pm$  SD. \*\*:  $p < 0.01$  vs. CD95L alone. (Dunnett's Multiple Comparison Test)

**Figure 4.** Time course of caspase activity **(A)** and cytotoxicity **(B)** of receptor mediated cell death in HepG2 and pmH cells in the presence of various HDIs, exemplarily depicted on HepG2 treated with CBHA/CD95L

Cells were treated with 5  $\mu$ M CBHA alone (open circle) or together with the ligands TRAIL (100 ng/ml; diamond shaped), CD95L (10% v/v; square) or TNF $\alpha$  (100 ng/ml; triangle). Cytotoxicity and caspase-3- like activity was determined at the indicated time points. Data represent means  $\pm$  SD.

**Figure 5:** Time course of the expression of the anti-apoptotic proteins cFLIP and XIAP upon exposure of HepG2 (left) and pmH (right) to HDIs.

Expression of cFLIP and XIAP was detected by Western blot analysis with specific antibodies using cell lysates from HepG2 **(left)** and pmH **(right)** exposed to CBHA **(upper panel)** and VPA **(lower panel)** for up to 21 h. Each lane was loaded with 30  $\mu$ g protein.

**Figure 6:** Influence of HDIs on extrinsic versus intrinsic pathway proximal to death-receptor induction

**(A)** HepG2 cells were transfected with either caspase-8 or with empty vector. Cells were treated with M344 (1  $\mu$ M), CBHA (5  $\mu$ M), apicidin (0.5  $\mu$ M) or VPA (12.5 mM). After 16 hours the caspase-3 like activity was measured by quantification of DEVD-afc cleavage.

+:  $p < 0.05$  vs. empty vector control \*\*:  $p < 0.01$  vs. caspase-8 control (Dunnett's Multiple Comparison Test)

**(B)** S100 extracts derivated from HepG2 cells treated with M344 (1  $\mu$ M), CBHA (5  $\mu$ M), apicidin (0.5  $\mu$ M) or VPA (12.5 mM) for 21 hours were incubated with 5  $\mu$ M cytochrome C and 1 mM dATP at 37°C. PETCM (200  $\mu$ M) was directly added into the S100 extracts 15 min prior addition of cytochrome c and dATP. After 30 min, caspase-3-like activity was determined. Data represent means  $\pm$  SD. \*:  $p < 0.05$  vs control \*\*:  $p < 0.01$  vs control (Dunnett's Multiple Comparison Test)

**Figure 7:** Influence of HDIs on intrinsic pathway of apoptosis induced by DNA damage

HepG2 (**A**) were preincubated with M344 (1  $\mu$ M), CBHA (5  $\mu$ M), apicidin (0.5  $\mu$ M) or VPA (12.5 mM). After 3 h, the cells were treated using increasing concentration of Melphalan and cytotoxicity was assessed 30 h later by LDH-release assay.

PmH (**B**) were preincubated with apicidin (5  $\mu$ M), M344 (100  $\mu$ M), CBHA (100  $\mu$ M), or VPA (12.5 mM). After 3 h, the cells were exposed to UV-radiation and cytotoxicity was assessed 21 h later by LDH-release assay. Data represent mean  $\pm$  SD.

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Table 1 Equipotent HDI concentrations for CD95L-induced cytotoxicity in HepG2 and pmH.

[ $\mu$ M]	Apicidin	M344	CBHA	VPA
HepG2	0.5	1	5	12500
pmH	5	100	20	12500

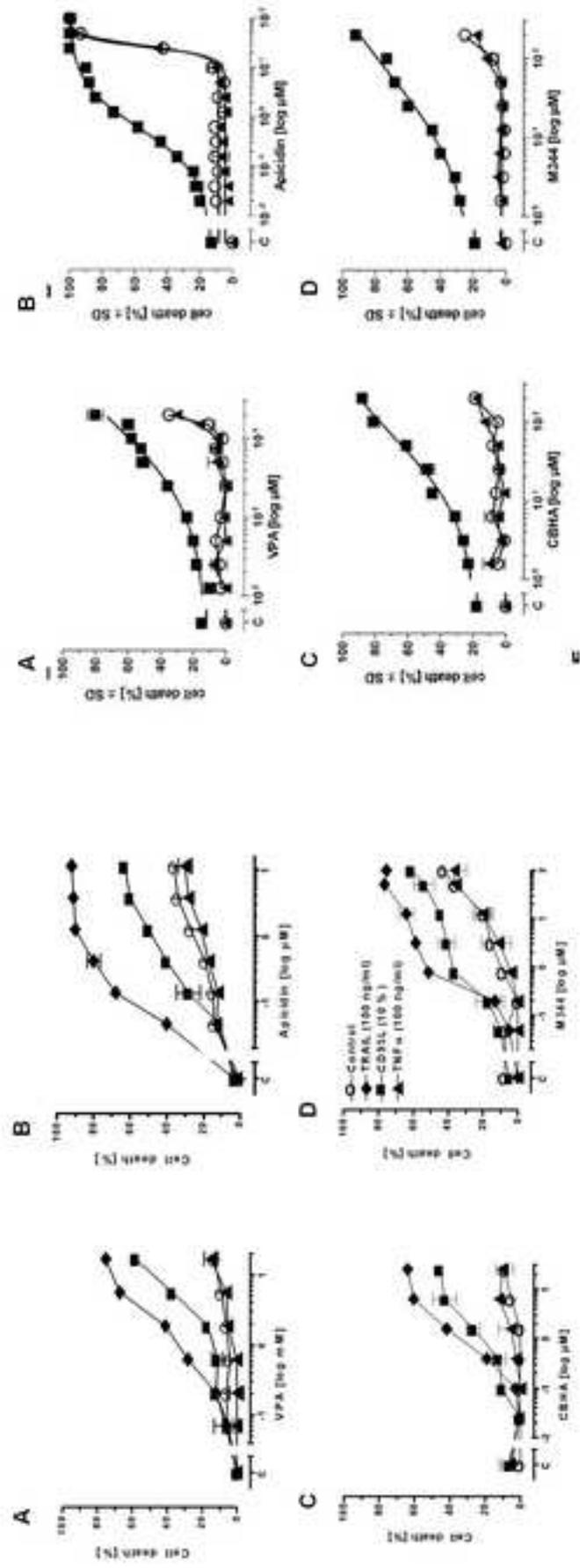


Figure 1

