

Histone deacetylase inhibition by valproic acid down-regulates c-FLIP/CASH and sensitizes hepatoma cells towards CD95- and TRAIL receptor-mediated apoptosis and chemotherapy

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Abstract. Hepatocellular carcinoma (HCC) is highly resistant to chemotherapy, leading to a poor prognosis of advanced disease. Inhibitors of histone deacetylase (HDACi) induce re-differentiation in tumor cells and thereby re-establish sensitivity towards apoptotic stimuli. HDACi are entering the clinical stage of tumor treatment, and several substances are currently being tested in clinical trials to prove their efficacy in the treatment of leukemias and solid tumors. In this study, we investigated the impact of the HDACi valproic acid (VA) on TRAIL- and CD95-mediated apoptosis in hepatoma cells, as well as its sensitizing effect on a chemotherapeutic agent. Treatment of HepG2 cells with VA increased sensitivity to CD95-mediated apoptosis (4% apoptosis vs. 42%), and treatment with epirubicin (74% vs. 90% viability). Caspase-3 activity was significantly enhanced in cells treated with VA plus anti-CD95 antibodies compared to cells treated with antibodies alone. In parallel, VA strongly augmented the effect of TNF-related apoptosis-inducing ligand (TRAIL or Apo2 ligand) on HepG2 cells (10% vs. 58% apoptosis). VA induced down-regulation of cellular FLICE-inhibitory protein (c-FLIP/CASH, also known as Casper/iFLICE/FLAME-1/CLARP/MRIT/usurpin), providing a possible molecular mechanism underlying the increased sensitivity towards cell death-mediated apoptosis. HDAC inhibitors are a promising class for the treatment of leukemias. In addition, among other class members, VA deserves further evaluation as a treatment option for patients with advanced HCC.

Introduction

HCC is a growing clinical problem, making it the fifth most common cause of cancer (1). Usually arising from a cirrhotic liver, with an additional disease-specific risk of malignant transformation, HCC cells are particularly resistant to chemotherapy. Although the enigma of this particular resistance is only partially understood, a large body of data suggests that the process of oncofetal dedifferentiation enables hepatocytes to evade immune surveillance by refining their intracellular set of proteins, which orchestrate the subtle balance of apoptotic death and survival. Evading the immune surveillance by developing resistance to apoptosis is a classic principle, which is of particular importance in a number of malignancies including HCC, where increased levels of anti-apoptotic proteins such as c-FLIP/CASH (2-4) [also coined as Casper/iFLICE/FLAME-1/CLARP/MRIT/usurpin (5-10)] and survivin (11) or decreased levels of pro-apoptotic proteins such as FADD (12) have been described.

In clinical practice, only a subgroup of patients with early disease qualifies for curative treatment by resection or liver transplantation. In patients with progressive disease, restriction to the liver allows palliative treatment by transarterial chemoembolization. However, the chemoresistant nature of the tumor leads to a poor outcome for patients when the disease is advanced and extrahepatic dissemination of HCC has already occurred (13). Improved efficacy of chemotherapy is desperately needed. In this context, there is a growing interest in the concept to integrate the group of histone deacetylase inhibitors (HDAC-I) in chemotherapy protocols for patients with malignancies (14). Indeed, the inhibition of histone deacetylase by sodium butyrate or trichostatin A significantly enhanced the sensitivity of hepatoma cells to apoptosis (15,16). Since sodium butyrate alone does not appear to be the ideal candidate due to its unfavorable pharmacokinetics, we tested the short chain fatty acid valproic acid (VA), an approved drug to treat patients with seizures, which demonstrated to have strong HDACi activity (17,18). It was previously shown that VA itself induces apoptosis in leukemia cells (19,20).

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The combined treatment of the hepatoma cell line HepG2 with VA and death receptor agonists led to a substantial increase of apoptosis induction. We observed 40-75% apoptotic cells with the combination of VA and death receptor agonist even in concentrations which by itself led to apoptosis in only less than 10% of the cells. Although the underlying mechanism has not yet been fully elucidated, down-regulation of the anti-apoptotic molecule c-FLIP/CASH might contribute.

Our data support the idea to further evaluate the efficacy of treatment strategies for patients with HCC that combine death receptor activating agents, either by conventional chemotherapy or by selective agonists, with HDAC inhibitors such as valproic acid.

Materials and methods

Cells. The human hepatoma cell line HepG2 (no. HB 8065; ATCC) was cultured in Dulbecco's modified Eagle medium containing 10% FCS, 1% glutamin, 1% PenStrep, 1% sodium pyruvate and 1% HEPES buffer (Gibco BRL).

Reagents. Valproic acid (Ergenyl and Sanofi-synthelabo) and epirubicin (Sigma, Germany) were purchased. Anti-APO-1 is an agonistic monoclonal antibody (IgG3, k) recognizing an epitope on the extracellular part of CD95 (APO-1/Fas) (21). The anti-c-FLIP/CASH monoclonal antibody NF6 (mouse IgG1) was previously described (22). Anti-APO-1 and NF6 were gifts from Peter H. Krammer, DKFZ, Heidelberg, Germany.

Cell death assay. Cell viability was measured in the neutral red assay as previously described. Briefly, cells were seeded in 96-well plates. After the indicated time, cells were incubated in medium containing 1% neutral red (Sigma) for another 2 h, then washed 3 times before being lysed in Sorenson buffer as described (23). Absorbance was measured at 490 nm using an ELISA reader. To measure the extent of apoptosis, cells were seeded in 24-well plates. After the indicated time, apoptosis was measured by sub-G1 DNA content by FACS analysis as described (24).

Western blot analysis. Western blot analysis was performed with whole liver extract lysed in lysis buffer; protein concentration was equilibrated using the Bradford assay reagent. The samples were boiled in sodium dodecyl sulfate sample buffer and run on a 10% SDS-PAGE gel electrophoresis. Following electrophoresis, the samples were blotted to a PVDF membrane (Pall, Germany), and the membrane was blocked with 2% milk powder for 30 min following overnight incubation with the monoclonal anti-FLIP antibody at 4°C in phosphate-buffered saline/Tween 0.1% with 2% milk powder (PBSTM). After washing, membranes were incubated for 45 min with horseradish peroxidase-conjugated rabbit anti-mouse serum (dilution 1:3000) in PBSTM. Blots were developed using the Western Lightning chemiluminescence reagent (Perking-Elmer Life Sciences, Boston, MA, USA).

Caspase-3 activity. Snap-frozen cells were lysed in buffer containing 20 mM Tris/HCl pH 8.0, 5 mM EDTA, 0.5% Triton X-100 and Complete® protease inhibitor cocktail (Boehringer). Protein concentration was equilibrated by

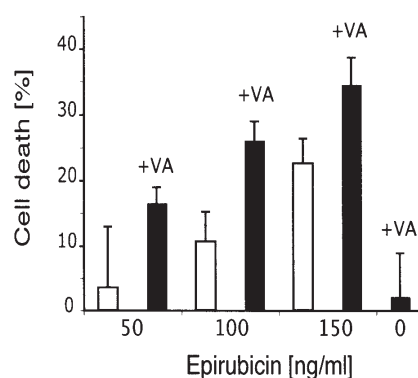


Figure 1. Valproic acid augments epirubicin-induced cell death. HepG2 cells were treated with 100 μ g/ml valproic acid (VA) and epirubicin (Epi) for 48 h. The viability of cells was quantified by neutral red assay.

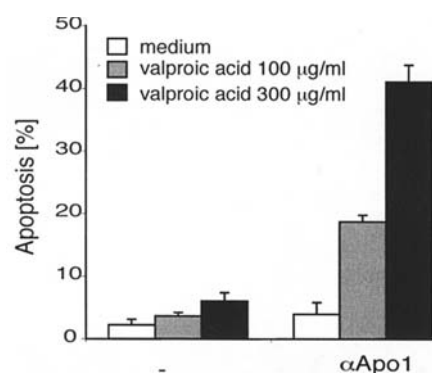


Figure 2. Valproic acid augments CD95-induced apoptosis. HepG2 cells were treated with 100 μ g/ml valproic acid (VA) and anti-CD95 antibodies (200 ng/ml anti-Apo1 and 10 ng/ml protein A) for 48 h. Apoptosis was determined by FACS analysis.

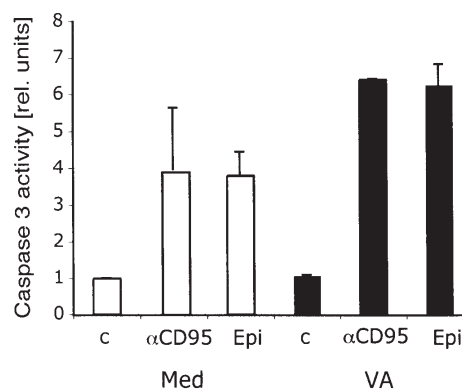


Figure 3. Caspase-3 activation in HepG2 cells. Cells were incubated for 48 h. Control (c), epirubicin (Epi; 150 ng/ml), anti-CD95 antibodies anti-Apo-1 (α CD95; 200 ng/ml) and protein A (10 ng/ml), and valproic acid (VA; 100 μ g/ml).

Bradford assay (Sigma). Lysates were incubated in reaction buffer (25 mM HEPES pH 7.5, 50 mM NaCl, 10% glycerol, 0.05% CHAPS, and 5 mM dithiothreitol) in the presence of 50 mmol/l fluorogenic substrates (Biomol, Germany) preferentially cleaved by caspase-3 (DEVD-AFC). The fluorometric assay was performed on microtiter plates (Greiner, Germany), and the generation of free AFC at 37°C after 1 h was measured using a fluorometer plate reader (Tecan, Germany) set at an excitation wavelength of 405 nm and an emission wavelength of 535 nm. Albumin secretion

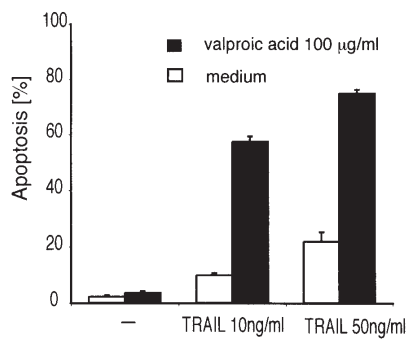


Figure 4. Valproic acid augments TRAIL-induced apoptosis. HepG2 cells were treated with 100 µg/ml valproic acid (VA) and TRAIL for 24 h. Apoptosis was determined by FACS analysis.

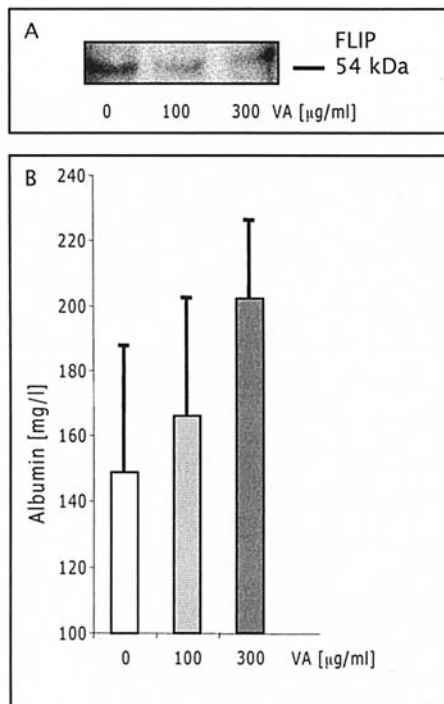


Figure 5. (A) Down-regulation of the caspase-8 homologue c-FLIP/CASH in HepG2 cells by valproic acid (VA) treatment for 48 h. (B) Re-differentiation of HepG2 cells by VA led to an increase of albumin production.

was measured using the automatic nephelometer from Dade Behring, BNA, BN II.

Results

We used valproic acid (VA), an approved antiepileptic drug, to further investigate the idea of increasing the efficacy of apoptosis-inducing chemotherapeutic drugs. VA did not significantly activate the apoptotic cascade in HepG2 cells in approved therapeutical concentrations by itself. However, it enhanced the cytotoxicity of epirubicin treatment up to 3-fold (Fig. 1). In order to evaluate whether this effect of VA is due to an increased sensitivity of hepatoma cells towards death receptor-mediated apoptosis, we first investigated the impact of VA treatment on CD95-induced apoptosis (Fig. 2): VA significantly increased the CD95-induced apoptosis in HepG2 cells and led to apoptosis of 42% of cells with 300 µg/ml VA and 19% of cells in the presence of 100 µg/ml VA compared

to 4% when treated with APO-1 alone. The apoptosis-augmenting effect of VA was accompanied by an increase in caspase-3 activity with CD95 or epirubicin, while VA alone did not significantly activate caspase-3 (Fig. 3). Interestingly, VA also sensitized HepG2 cells to other death receptors. TRAIL-induced apoptosis was dramatically increased by concomitant treatment with VA (16% vs. 59% apoptosis for 50 ng/ml TRAIL and 100 µg/ml VA, respectively) (Fig. 4).

To further evaluate the underlying mechanism, we analyzed the expression of the caspase-8-homologue c-FLIP/CASH, which has been well described as an anti-apoptotic protein. VA treatment decreased the c-FLIP/CASH expression level, thus laying the ground for a facilitated activation of the death receptor-triggered cascade of the apoptotic program (Fig. 5a). In addition, down-regulation of c-FLIP/CASH was accompanied by the re-differentiation of HepG2 cells, as demonstrated by a concentration-dependent increase of albumin secretion to the supernatant (Fig. 5b).

Discussion

Resistance to chemotherapy is a major obstacle in the treatment of advanced HCC. Hence, new regimens or improved efficacy of existing chemotherapy protocols are highly desirable. There is growing evidence that the class of HDAC inhibitors might contribute to increased efficacy of standard chemotherapy (14,25). Here, we demonstrate that VA sensitizes hepatoma cells towards apoptotic stimuli.

VA strongly enhanced the cytotoxic effect of the chemotherapeutic drug epirubicin. This enhancement was observed with a drug concentration equivalent to recommended serum levels in patients treated for seizures, which did not lead to cellular toxicity by itself. To clarify the involved mechanism, we investigated whether a direct and distinct apoptotic stimulus, such as stimulation of the death receptor CD95 by agonistic antibodies, is also enhanced by VA treatment. We were able to show that VA strongly enhanced CD95-induced cell death and caspase-3 activity. It is tempting to conclude that VA sensitizes the hepatoma cell line HepG2 towards epirubicin by sensitizing the cells towards receptor-mediated apoptosis, as was described in other cells with different HDACi (15,26). This view is supported by the observation that VA treatment down-regulated the anti-apoptotic caspase-8-homologue c-FLIP/CASH, which was shown to be a major regulator of TRAIL-induced apoptosis (27). In addition, treatment with VA led to increased albumin production in HepG2 cells, reflecting a state of re-differentiation of the hepatoma cells.

A previous study demonstrated that VA induces apoptosis by itself at higher concentrations (28). However, in concentrations equivalent to approved serum levels for treatment of seizures, we did not observe significant apoptosis induction.

Our observations support the idea to test VA in combination with chemotherapeutic drugs such as the anthracyclin epirubicin in the treatment of advanced HCC. The addition or substitution of classical chemotherapy with death receptor-activating agents such as TRAIL is extremely tempting and ready to enter the clinical stage. While TNF and the Fas ligand proved to be highly toxic to human hepatocytes, and despite initial data suggesting a direct toxic role for TRAIL ligand (29), there is growing evidence that the specifically

triggering TRAIL receptor is not toxic to non-malignant primary hepatocytes (30-32) and might have a role in upcoming regimens of cancer therapy (33). In conclusion, our data further substantiate the need to evaluate combination therapies with agents sensitizing towards apoptosis and those inducing its execution, and VA and TRAIL appear to be a promising pair.

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