HDAC Inhibitor Treatment of Hepatoma Cells Induces Both TRAIL-Independent Apoptosis and Restoration of Sensitivity to TRAIL

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Hepatocellular carcinoma (HCC) displays a striking resistance to chemotherapeutic drugs or innovative tumor cell apoptosis–inducing agents such as tumor necrosis factor–related apoptosis-inducing ligand (TRAIL). Recently, we found 2 histone deacetylase inhibitors (HDAC-I), valproic acid and ITF2357, exhibiting inherent therapeutic activity against HCC. In TRAIL-sensitive cancer cells, the mechanism of HDAC-I–induced cell death has been identified to be TRAIL-dependent by inducing apoptosis in an autocrine fashion. In contrast, in HCC-derived cells, a prototype of TRAIL-resistant tumor cells, we found a HDAC-I-mediated apoptosis that works independently of TRAIL and upregulation of death receptors or their cognate ligands. Interestingly, TRAIL resistance could be overcome by a combinatorial application of HDAC-I and TRAIL, increasing the fraction of apoptotic cells two- to threefold compared with HDAC-I treatment alone, whereas any premature HDAC-I withdrawal rapidly restored TRAIL resistance. Furthermore, a tumor cell–specific downregulation of the FLICE inhibitory protein (FLIP) was observed, constituting a new mechanism of TRAIL sensitivity restoration by HDAC-I. In contrast, FLIP levels in primary human hepatocytes (PHH) from different donors were upregulated by HDAC-I. Importantly, combination HDAC-I/TRAIL treatment did not induce any cytotoxicity in nonmalignant PHH. In conclusion, HDAC-I compounds, exhibiting a favorable *in vivo* profile and inherent activity against HCC cells, are able to selectively overcome the resistance of HCC cells toward TRAIL. Specific upregulation of intracellular FLIP protein levels in nonmalignant hepatocytes could enhance the therapeutic window for clinical applications of TRAIL, opening up a highly specific new treatment option for advanced HCC. (HEPATOLOGY 2006;43: 425-434.)

At the time of first diagnosis, most hepatocellular carcinoma (HCC) patients present themselves in advanced disease stages, excluding them from any curative surgical approaches.[1] Unfortunately, HCC displays a particular resistance to cytostatic agents[2,3] in addition to innovative therapeutic approaches such as induction of tumor cell–selective apoptosis by the tumor necrosis factor–related apoptosis-inducing ligand (TRAIL).[4-6] Therefore, alternative treatment strategies are urgently needed for a large number of HCC patients worldwide.

Epigenetic cancer therapies such as the employment of histone deacetylase inhibitors (HDAC-I) reflect the discovery that many malignant diseases share an "epigenetic" etiology. HDAC-I induce proliferation arrest, differentiation, and apoptosis selectively in cancer cells but not in normal cells.[7-9] Interestingly, we and others could demonstrate that substances with HDAC-I activity induce a profound and tumor cell-selective apoptosis of hepatoma-

Abbreviations: HCC, hepatocellular carcinoma; TRAIL, tumor necrosis factor–related apoptosis-inducing ligand; HDAC-I, histone deacetylase inhibitor; FLIP, FLICE inhibitory protein; PHH, primary human hepatocyte; VPA, valproate; R1, receptor 1; R2, receptor 2; mRNA, messenger RNA; RT-PCR, reverse-transcriptase polymerase chain reaction.

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derived cell types without comparable effects in nonmalignant, human-derived primary hepatocytes[10-12] or by priming hepatoma cells for the innate immune system.[13]

To date, the molecular mechanisms of HDAC-I activity in different tumor cell types are only partially understood. Two recent reports (using TRAIL-sensitive tumor cell types such as leukemic cells) suggested that the tumor specific response to HDAC-I is mediated by TRAIL in an autocrine fashion (Fig. 1A).[14,15] However, several tumor types including HCC are known to exert a profound inherent resistance to a treatment with TRAIL, mostly as a result of constitutive expression of intracellular TRAIL resistance mediating factors, such as FLIP[16] (Fig. 1B). Nevertheless, HCC-derived cell types, representing a prototype for TRAIL-resistant tumor cells, undergo apoptosis in response to treatment with HDAC-I.[10] Consequently, substances with HDAC-I activity are expected to become valuable tools in cancer therapy as single agents or in combination treatment approaches.

In the current study, we found that HDAC-I treatment does not lead to an increase of proapopotic death receptor ligands nor their cognate receptors in HCC-derived cells. Unexpectedly, we observed an increased sensitivity to TRAIL itself, mediated by a HDAC-I–induced downregulation of the cellular apoptosis-modifying protein FLIP. In contrast, normal primary human hepatocytes (PHH) from different donors responded to HDAC-I treatment via upregulation of FLIP proteins. Thus, a combination treatment approach of HDAC-I and TRAIL was well tolerated by PHH, whereas HCC-derived cell types displayed a dramatically increased rate of apoptotic cell death. Taken together, HDAC-I substances seem to constitute the long sought-after tumor selective sensitizers to TRAIL (Fig. 1C), offering innovative therapeutic perspectives for HCC.

Materials and Methods

Substances. ITF2357 (Italfarmaco, Milano, Italy), valproate (VPA) (Ergenyl, Sanofi-Synthelabo, Berlin, Germany). Soluble recombinant human TRAIL (TRAIL) comprising residues 114-281 (Calbiochem, Schwalbach, Germany) was used in a concentration of 100 ng/mL. Agonistic anti-Fas antibodies (CH11) were from Upstate Direct (Germany).

Cells. According to the guidelines of the charitable state-controlled foundation HTCR (Thasler, Cell and Tissue Banking 2003), PHH were kindly provided by T.S. Weiss, University of Regensburg, and cultured as described.[17] In brief, PHHs were cultured in DMEM (supplemented with 1.66 mU/mL insulin (Novo Nordisk, Bagsvaerd, Denmark), 3.75 ng/mL hydrocortisone

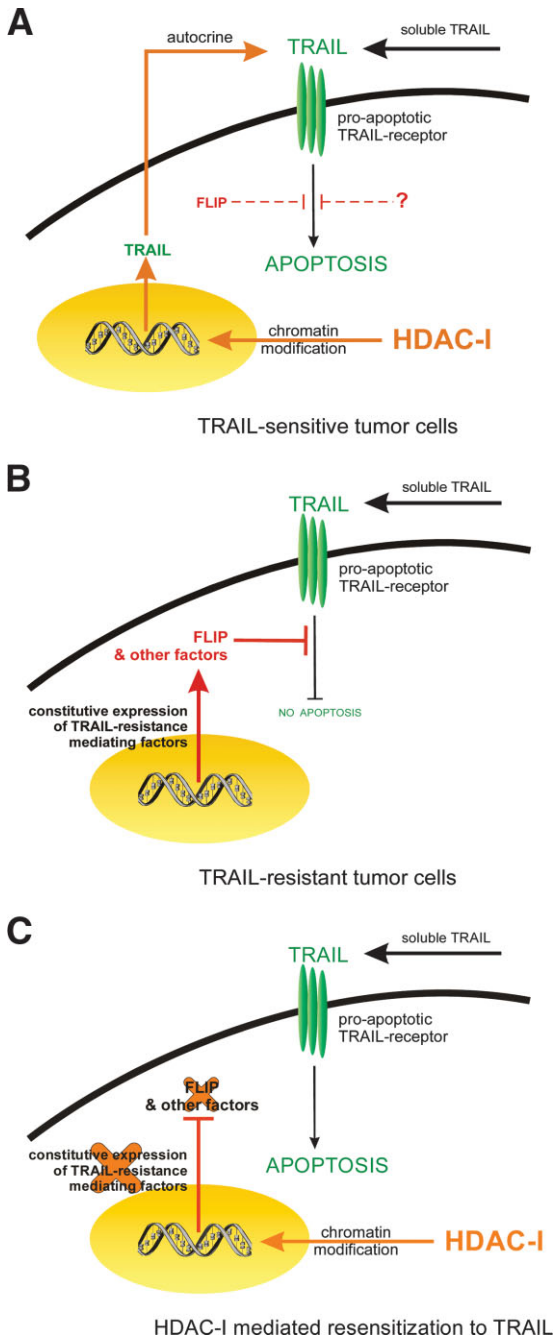

Fig. 1. HDAC-I–mediated modulation of TRAIL activity in TRAIL-sensitive or TRAIL-resistant tumor cells. (A) In TRAIL-sensitive tumor cells, HDAC-I have been shown to induce cell death by triggering an autocrine TRAIL-mediated apoptotic pathway.[14,15] (B) TRAIL-resistant tumor cells such as hepatoma cells are protected against TRAIL-induced apoptotic cell death by constitutive expression of TRAIL resistance mediating factors such as FLIP. (C) HDAC-I are able to resensitize TRAIL-resistant hepatoma cells not by altering the expression levels of TRAIL or TRAIL receptors but via downregulation of TRAIL resistance mediating factors such as FLIP. TRAIL, tumor necrosis factor–related apoptosis-inducing ligand; FLIP, FLICE inhibitory protein; HDAC-I, histone deacetylase inhibitor.

(Merck, Darmastadt, Germany), 2 mmol/L glutamine, and the antibiotics penicillin and streptomycin for 3 days after isolation as described.[17] Media exchange was performed daily. Experiments with HDAC-I were performed starting at day 4. Human hepatoma cell lines HepG2 and HuH7 were obtained from ATCC (Rockville, MD). HuH7 cells were maintained in DMEM- (+10% FCS), HepG2 cells in MEM and DMEM (3:1; +10% FCS). Media and supplements were obtained from Life Technologies (Rockville, MD). Cells were plated in 6 wells at 100,000 cells per well for 24 hours and treated with HDAC-I and/or TRAIL as specified.

Flow Cytometric Detection of Apoptosis and TRAIL Receptors R1 or R2. Fragmentation of genomic DNA to hypodiploid DNA was assessed by FACS analysis as previously described.[18] For receptor 1 (R1) or receptor 2 (R2) detection, cells were washed in ice-cold PBS containing 1% BSA and 0.05% sodium acid, detached with trypsin, and centrifuged for 5 minutes at 1,200*g*. Primary antibodies anti-TRAIL-R1 and anti-TRAIL-R2 (kindly provided by M. Weller, Tübingen, Germany) were added to the pellet and the mixture was incubated for 30 minutes at room temperature, followed by washing in PBS, centrifugation, and incubation with secondary antibody for 30 minutes at room temperature. After washing with PBS the pellet was incubated with streptavidin-PE for 30 minutes at room temperature. Flow cytometry (FACS Calibur; Becton Dickinson, Heidelberg, Germany) was performed using the CellQuest program.

Immunoblotting. Western blot assays were performed as previously described[19] with monoclonal anti-FLIP (1:1,000) (Bioscience, Vienna, Austria) and monoclonal anti-Vinculin (Sigma, Munich, Germany).

Cytotoxicity and Caspase Assay for PHHs. Culture media from PHH and HepG2 cells were screened for enzyme activities of alanine aminotransferase and lactate dehydrogenase using commercial ELISA procedures (ADVIA 2400 analyzer; Bayer ADVIA, Fernwald, Germany). The enzymatic activities in supernatants were related to untreated cultures. Experiments were performed in quadruplicate and PHH were employed from 5 different donors. Caspase-3-like activity was performed as described[18]. One-way ANOVA with Dunnett's posttest was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA).

Quantitative Reverse-Transcriptase Polymerase Chain Reaction. Gene expression of defined genes from HepG2 cells and PHH was quantified using Assays on Demand (Applied Biosystems, Darmstadt, Germany). Briefly, first- and second-strand complementary DNA was synthesized from 5 μg of total RNA using the SuperScript Double-Stranded cDNA Synthesis Kit (Life Technologies) and random hexamer primers as suggested by the manufacturer. Assays on Demand were used for the relative quantitation of FLIP (Hs00153439), TRAIL (Hs00269492), TRAIL-R1 (Hs00187196), TRAIL-R2 (Hs00234355), FAS (Hs00163653), FAS-L (Hs00181225), and GAPDH (Hs99999905). Assays were performed in duplicate. The ΔC_T value was determined by subtracting the average RNA C_T value from the average FLIP C_T value. The relative change of expression was calculated as $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T$ is the difference of ΔC_T from cells treated with HDAC-I versus untreated cells. The standard deviation of the ΔC_T was calculated using the formula $s = (s_1^2 + s_2^2)$, where s_i is the standard deviation of the calculated C_T values.

Microarray Expression Analysis. Gene expression profiles of HepG2 cells cultured for 24 hours with or without 1 mmol/L VPA or 0.2 μmol/L ITF2357 were determined using Affymetrix microarray analysis and the human genome HU133A oligonucleotide array chip (Affymetrix, Santa Clara, CA) as previously described.[13] The signal intensity for each gene was calculated as the average intensity difference, represented by Σ [(PM − MM)/(number of probe pairs)], where PM and MM denote perfect match and mismatch probes. The messenger RNA (mRNA) of a gene was considered expressed ("present") or changed when the detection *P* value and change *P* value were less than .05. All microarray data shown are the results of 3 independent experiments with single RNA labeling and hybridization.

Results

HDAC-I–Induced Death of Hepatoma-Derived Cells Is Not Mediated via TRAIL. Two recent reports have suggested TRAIL as the main mediator of HDAC-I activity in tumor cells.[14,15] However, these observations have been made in TRAIL-sensitive leukemia-derived cell types. Interestingly, HDAC-I-induced specific tumor cell death has also been described in cells displaying a profound resistance to exogenously added TRAIL.[10] In this context, it was tempting to speculate whether a HDAC-I-mediated sensitization to TRAIL of tumor cells with an inherent TRAIL resistance, such as hepatoma-derived cells, is affected by an autocrine, TRAIL-induced cell death mechanism similar to the one found for TRAIL-sensitive tumor cells.[14,15]

To investigate this hypothesis, the HCC cell lines HepG2 and HuH7 were treated with HDAC-I compounds VPA or ITF2357[7] and examined for a potential transcriptional upregulation of TRAIL, DR5, Fas Ligand, or Fas, being indicative for a putative HDAC-I-mediated upregulation of key death receptors or their cognate li-

Table 1. Microarray Analysis of Apoptosis Signalling Components in HDAC-I Treated HepG2

		Control Cells		VPA Treated Cells			ITF Treated Cells		
GeneBank ID[a]	Gene Name	Signal[b]	Detection[c]	Signal[b]	Detection[c]	Fold Change[d]	Signal[b]	Detection[c]	Fold Change[d]
U57059.1	TRAIL	31 ± 1	P	36 ± 9	P	NC	48 ± 16	P	NC
AF016266.1	TRAIL-R1	465 ± 16	P	495 ± 72	P	NC	508 ± 55	P	NC
AF288573.1	FasL	35 ± 119	A	37 ± 20	A	NC	25 ± 9	A	NC
NM_000043.1	Fas	124 ± 1	P	81 ± 27	M	NC	91 ± 12	P	NC
AF005775.1	FLIP	584 ± 10	P	262 ± 35	P	0.45	345 ± 35	P	0.6
M33197.1	GAPDH[e]	3035 ± 300	P	2959 ± 194	P	NC	3143 ± 290	P	NC

Note. Gene expression profile of HepG2 cells (control cells) and HepG2 cells cultured in the presence of either 1 mmol/L VPA (VPA treated cells) or 0.2μmol/L ITF2357 (ITF treated cells).

[a]Gene bank accession number.

[b]Calculated mean fluorescence intensity signal on Human Genome HU133A oligonucleotide array chip.

[c]Classification of gene expression as absent (A), present (P) or marginal (M), according to the calculation of the scores of 11 perfect match and of 11 mismatch probes for the same hybridized gene using the Microarray Suite software.

[d]Proportion of the signals from experiments with VPA or ITF2357 treated cells to untreated control cells for genes with changed expression (fold change >1 for increased and <1 for decreased gene expression; NC for "no change").

[e]GAPDH control as a housekeeping gene that is highly transcribed an is not influenced by HDAC-I treatment.

gands. As a result, no significant upregulation in the transcript levels of these genes were detected (Table 1). Importantly, this finding has been validated by additional experiments employing quantitative RT-PCR analysis (data not shown).

We next performed FACS analysis and real-time PCR to assess whether either VPA or ITF2357 treatment increases expression of proapoptotic TRAIL receptors (TRAIL-R1/-R2) or their transcripts. However, no substantial upregulation of hepatoma cell surface expression (Fig. 2A-E) or transcript levels (Fig. 2G) of TRAIL-R1/-R2 could be detected. Taken together, our findings suggest that HDAC-I-mediated apoptotic cell death of human hepatoma cells exhibiting an inherent TRAIL resistance is not induced via TRAIL-mediated autocrine cell killing. This is in sharp contrast to the recent reports investigating TRAIL-sensitive leukemic cells.[14,15]

HDAC-I Compounds Prime Human Hepatoma Cells to TRAIL-Induced Apoptosis. Numerous studies have shown a substantial genetic reprogramming of cancer cells in the course of HDAC-I treatment.[7-10] Thus, we next investigated whether HDAC-I can alter the reaction pattern of HepG2 or HuH7 human hepatoma cells to exogenously supplemented (soluble) TRAIL. Surprisingly, when TRAIL was added during the last 24 hours of a 48-hour HDAC-I incubation period, we noted a dramatic increase in cell death rates up to threefold (Fig. 3A-B, black bars). However, maximum TRAIL-enhanced tumor cell death required permanent presence of HDAC-I: sequential incubation (*e.g.*, first with either VPA or ITF2357 for 24 h, then with TRAIL solely for a further 24 hours) did not lead to a substantial increase in the rate of apoptotic cells (Fig. 3C, white bars) when compared with a continuous 48-hour treatment of

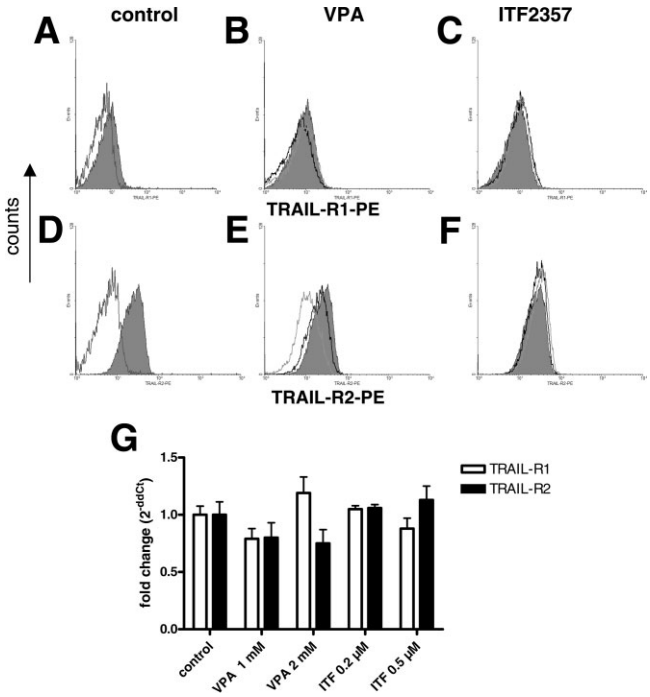

Fig. 2. HDAC inhibition does not increase cell surface expression of proapoptotic TRAIL receptors (TRAIL-R1/-R2) in human hepatoma cells. HepG2 cells were treated with VPA or ITF2357 for 48 hours. Cells were labeled with biotinylated antibodies against (A-C) TRAIL-R1 or (D-F) TRAIL-R2 together with streptavidin-PE. (A,D) Filled histograms represent cell surface expression of either TRAIL-R1 or -R2; unfilled histograms represent results of isotype control experiments. (B,E) Treatment was performed with 1 mmol/L (full line) or 2 mmol/L VPA (gray line). Filled histograms represent untreated cells. (C,F) Treatment was performed with 0.2 μmol/L (full line) or 0.5 μmol/L (gray line) ITF2357. Filled histograms represent untreated cells. (G) Quantitative TRAIL-R1 (white bars) and TRAIL-R2 (black bars) RT-PCR from HepG2 cells either incubated for 48 hours with VPA or ITF2357 relative to untreated cells (control). VPA, valproate; TRAIL, tumor necrosis factor–related apoptosis-inducing ligand; R1, receptor 1; PE, phycoerythrin; R2, receptor 2; ITF, ITF2357.

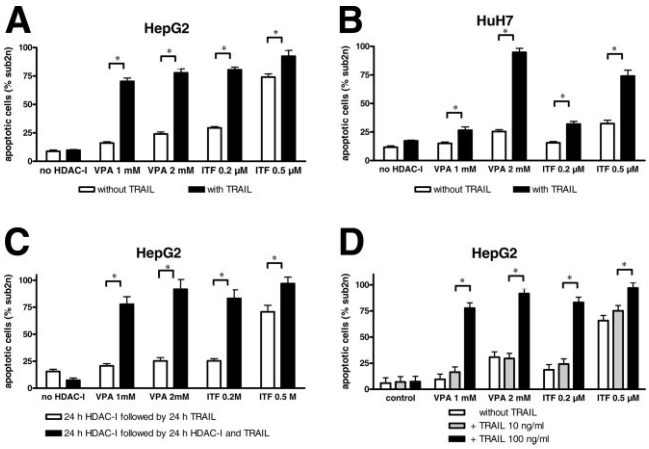

Fig. 3. TRAIL sensitization of human hepatoma cells by HDAC-I. (A) HepG2 or (B) HuH7 cells were incubated with different HDAC-I compounds (VPA or ITF2357) for 48 hours at different concentrations with (black bars) or without (white bars) supplementation of TRAIL for the last 24 hours of the incubation period. The amount of apoptosis was measured by FACSanalysis (sub2n-DNA quantification). Shown are the means of 2 independent experiments including single standard deviation. (C) HepG2 cells were incubated with HDAC-I substances either continuously for 48 hours (black bars) or for the first 24 hours of the experiment only (white bars). Recombinant soluble TRAIL was added during the last 24 hours of the experiment either in the presence of a HDAC-I compound (black bars) or alone (white bars). (D) HepG2 cells were treated with VPA or ITF2357 for 48 hours and TRAIL was added in 2 different concentrations (10 or 100 ng/mL) for the last 24 hours of the incubation period. The rate of apoptosis was determined via fluorescence-activated cell sorting (sub2n-DNA quantification). Shown are the means of 2 independent experiments including single standard deviation. $*P < .05$. HDAC-I, histone deacetylase inhibitor; VPA, valproate; ITF, ITF2357; TRAIL, tumor necrosis factor–related apoptosis-inducing ligand.

HDAC-I (Fig. 3C, black bars). Looking at the concentration of TRAIL, we found that low doses (*e.g.*, 10 ng/mL) were not sufficient for the observed combinatorial treatment effect (Fig. 3D).

Subsequently, performing a Western blot analysis on the acetylation pattern of HDAC-I–treated hepatoma cells, we found that both VPA and ITF2357 induced a marked hyperacetylation of H3-histone proteins not only at 24 hours, but also after a prolonged incubation period of 48 hours (data not shown). However, when HDAC-I was withdrawn after 24 hours, acetylation was found to be rapidly reduced to baseline levels at 48 hours (data not shown). Concluding from these observations, we hypothesized that a change of the acetylation status is followed by similar change in the expression level of one or more distinct intracellular apoptosis-modulating proteins, playing a key role in TRAIL-resistance of human hepatoma cells.

Differential Regulation of FLIP in Human Hepatoma Cells and PHHs. To further elucidate fundamental mechanisms of the HDAC-I induced TRAIL-sensitization of human hepatoma cells, we next considered intracellular apoptosis-modulating proteins as potential key regulators. In this context, expression of the FLICE-inhibitory protein FLIP is known to inhibit the upstream activation of caspase 8 and already has been identified as a major factor in the regulation of cell death and survival in HCC patients.[16] For this purpose, HepG2 cells were incubated with different concentrations of VPA or ITF2357 for 24 hours or 48 hours followed by total RNA preparation required for subsequent FLIP-specific quantitative RT-PCR analysis. As a result, HepG2 cell incubation with VPA led to a considerable decrease of the basic FLIP mRNA level after VPA or ITF2357 incubation for 24 or 48 hours (Fig. 4A, left side).

Next, we investigated whether this incubation regime (VPA or ITF2357 for 24 h or 48 h) leads to similar changes in cellular FLIP protein levels. As a result of additional Western blot analysis, a substantial downregulation of FLIP was found in the course of a 24-hour (Fig. 5A) incubation period and even more pronounced during a 48-hour (Fig. 5B) incubation period with either VPA or ITF2357. Interestingly, the results of both the FLIP protein level analysis (Fig. 5A-B) as well as FLIP-specific quantitative RT-PCR analysis (Fig. 4A, left side) were found to correlate quite well with the cytotoxicity data of identically treated cells as shown in Fig. 3C.

Having demonstrated that HDAC-I compounds selectively induce apoptosis in hepatoma cells but not in PHH,[10] we now further assumed that HDAC-I–promoted TRAIL sensitization might be based primarily on tumor-specific transcriptional changes. Therefore, we next evaluated FLIP expression levels in the PHH of 5 different donors over the course of VPA or ITF2357 exposure by quantitative RT-PCR analysis. In contrast to hepatoma-derived HepG2 cells, PHH demonstrated an increase of FLIP mRNA levels under HDAC-I treatment (Fig. 4A, right side). In addition, these mRNA data were found to be consistent with Western blot analysis, which showed a dose-dependent decrease of FLIP protein levels in response to VPA or ITF2357 in HepG2 and HuH7 cells (Fig. 4B, left and middle panel), but an upregulation of FLIP in PHH following HDAC-I exposure (Fig. 4B, right panel).

To further examine whether the observed changes of FLIP in PHH cells also induce functional changes, we next incubated PHH cells with agonistic anti-Fas antibodies (CH11). First, we could detect a profound induction of apoptotic cell death, which is reflected by a time-dependent increase of caspase-3-like activity in cellular lysates (Fig. 6A). Next, we investigated whether a 48-hour preincubation period with HDAC-I was able to modulate apoptosis induction in PHH. Strikingly, for both VPA and ITF2357 we noted a significant reduction in caspase

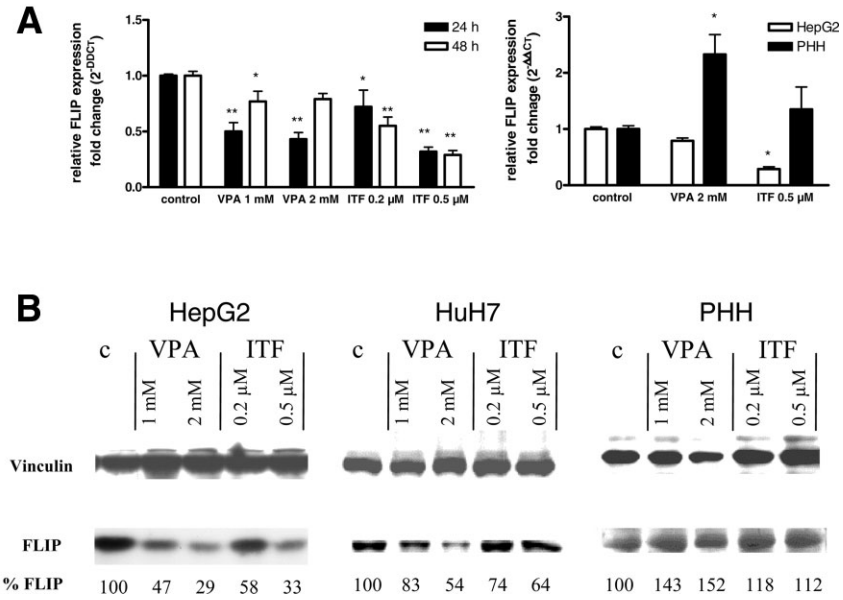

Fig. 4. Differential FLIP regulation pattern in human-derived malignant hepatoma cells and primary hepatocytes. (A, left side) Quantitative FLIP RT-PCR using HepG2 cells at two different time points after the beginning of HDAC-I incubation with VPA or ITF2357. (A, right side) Quantitative FLIP RT-PCR using HepG2 cells (white columns) or PHH (black columns) incubated for 48 hours with 2 mmol/L VPA, 0.5 μmol/L ITF2357, or growth medium only (control). Shown are the means of three independent experiments including single standard deviation. *$P < .05$. **$P < .001$. (B) Western blot analysis using antibodies against FLIP performed on HepG2 cells, HuH7 cells, or PHH treated for 48 hours with 1 mmol/L or 2 mmol/L VPA, 0.2 μmol/L or 0.5 μmol/L ITF2357, or growth medium only. FLIP levels were estimated by performing a densitometric analysis; values are shown relative to untreated controls. FLIP, FLICE inhibitory protein; VPA, valproate; ITF, ITF2357; PHH, primary human hepatocyte; c, control.

activity (Fig. 6B) as well as in lactate dehydrogenase release into the supernatant (Fig. 6C).

Taken together, these experiments suggest that the ability of HDAC-I compounds VPA and ITF2357 to reduce FLIP expression might contribute substantially to the phenomenon of sensitizing human hepatoma cell lines to TRAIL-mediated apoptosis. Furthermore, the completely different modulation pattern of intracellular FLIP expression in human hepatoma cells versus untransformed healthy human hepatocytes suggests a tumor-specific TRAIL sensitization by substances with HDAC-I activity.

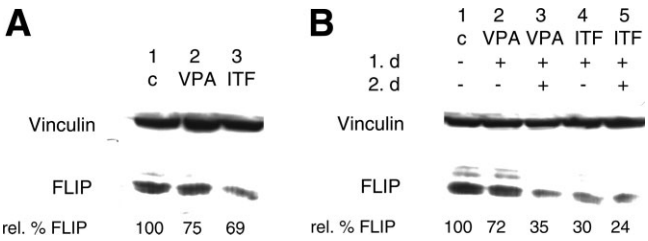

Fig. 5. Time course of FLIP levels in hepatoma cells. Two different settings were chosen to explore the time course of HDAC-I–mediated FLIP levels in HepG2 cells: (A) incubation for 24 hours either with medium alone (lane 1) or in the presence of 1 mmol/L VPA (lane 2) or 0.2 μmol/L ITF2357 (lane 3); (B) ongoing incubation for 48 hours either with medium alone (lane 1) or in the presence of 1 mmol/L VPA (lane 3) or 0.2 μmol/L ITF2357 (lane 5). As a control in (B), an incubation with VPA (lane 2) or ITF2357 (lane 4) restricted to the first 24 hours was added, followed by a further 24-hour period without any HDAC-I in the supernatant. Western blotting was performed employing antibodies against FLIP; equal protein loading was verified via vinculin quantification on the same blot (upper row). FLIP levels were estimated performing a densitometric analysis; values are shown relative to untreated controls (A-B, lane 1). VPA, valproate; ITF, ITF2357; FLIP, FLICE inhibitory protein.

HDAC-I/TRAIL Treatment Does Not Impose Any Toxicity on PHH. Since the discovery that certain recombinant TRAIL preparations can lead to a massive apoptosis of liver cells,[20-22] each new application strategy of TRAIL has to be tested extensively for potential liver toxicity. Consequently, the observation of a differentially (tumor vs. normal cell) HDAC-I–regulated FLIP expression led us to explore the potential cytotoxic effects of a combinatorial HDAC-I/TRAIL exposure (48 h HDAC-I ± TRAIL for the last 24 h) on the PHH of five different donors (Fig. 7A) versus HepG2 hepatoma cells (Fig. 7B). According to our previous results, HepG2 cells showed a marked increase in alanine aminotransferase release into cellular supernatants even when treated by HDAC-I substances only (Fig. 7B), whereas lactate dehydrogenase release—taken as a surrogate marker for severe cellular damage—was only detected in substantial amounts when HDAC-I substances and TRAIL were employed together (Fig. 7B). In sharp contrast to these observations, PHH did not show any signs of toxicity either using HDAC-I alone or in combination with TRAIL (Fig. 7A).

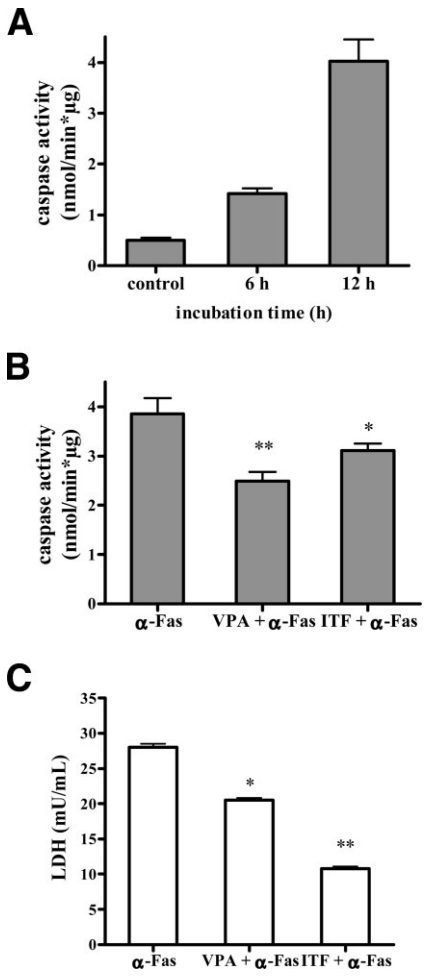

Fig. 6. HDAC-I–mediated desensitization of primary hepatocytes to Fas-induced apoptosis. (A) PHH were incubated with 100 ng/mL of an agonistic mouse–anti-Fas antibody. At the indicated time points, caspase-3-like activity was determined via fluorimetric caspase activity assay. (B-C) Forty-eight hours posttreatment of PHH with either 2 mmol/L VPA or 0.5 μmol/L ITF2357 media was changed with fresh HDAC-I and 100 ng/mL anti-Fas antibody was added. Caspase activity in cellular lysates (B) and lactate dehydrogenase release to the supernatant (C) were monitored 12 hours later. Shown are means of two independent experiments including single SD; $*P < .05$, $**P < .01$ compared with untreated control cells (no HDAC-I). VPA, valproate; ITF, ITF2357; LDH, lactate dehydrogenase.

This differential regulation pattern in human hepatoma-derived cell types versus PHH suggests a most promising safety profile for future combinatorial HDAC-I/TRAIL treatment regimes that opens a way to safe and highly effective treatment strategies in the fight against HCC.

Discussion

Since its first description in 1997, the cytokine TRAIL has been regarded as a promising substance in the field of innovative cancer therapy. This view is based mainly on the profound toxicity that TRAIL exerts on nearly all cancer cell types, which was found not to be effective for a wide array of healthy nonmalignant cell types.[4,23] Thus, TRAIL has been proposed as a highly effective and highly cancer cell–selective new compound and is currently being tested in clinical phase I studies.

Unfortunately, human hepatoma-derived cells have been found to be one of the exceptions to this general rule by exhibiting a major resistance to TRAIL-induced cell death.[18,23-25] Therefore, great efforts have been made to discover new principles that could help to sensitize HCC-derived cell types for the benefits of TRAIL-induced cell death.

In the present study, we succeeded in combining human hepatoma cell selective TRAIL-induced apoptosis together with the application of one of the most promising groups of new substances in investigational oncology, namely histone deacetylase inhibitors (HDAC-I).[8,9,26,27] Recently, we and others have suggested the use of HDAC-I as a potential new treatment option for HCC, due to its selective induction of apo-

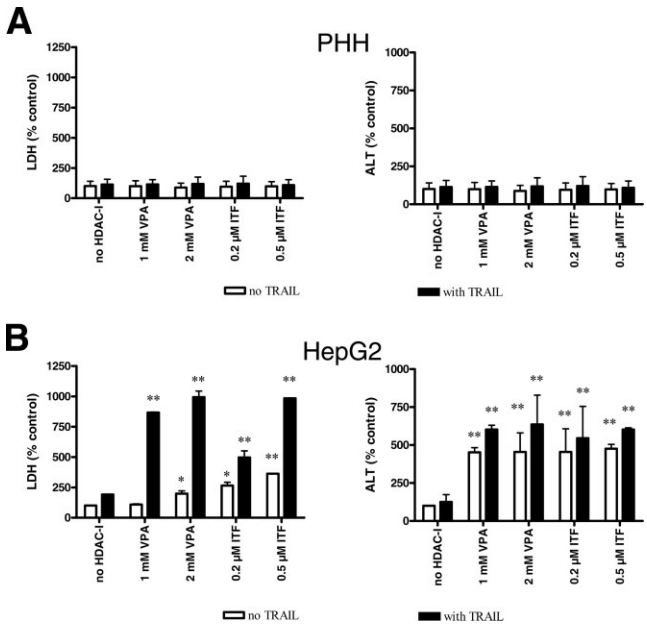

Fig. 7. Differential reaction pattern of human-derived hepatoma cells and primary hepatocytes during a combinatorial HDAC-I/TRAIL treatment. PHH from 5 different donors and HepG2 cells were cultured with VPA or ITF2357 for 48 hours at different concentrations with (white bars) or without (black bars) supplementation of TRAIL during the last 24 hours of the incubation period. Subsequently, release of (A,C) lactate dehydrogenase or (B,D) alanine aminotransferase into the supernatant was examined via ELISA. All values (% control) were referred to that of untreated control cells (control cells: 100%). Shown are the means of four independent experiments (for each PHH donor and for HepG2 cells), including single standard deviation. $*P < .05$, $**P < .01$ compared with untreated control cells (no HDAC-I). LDH, lactate dehydrogenase; HDAC-I, histone deacetylase inhibitor; VPA, valproate; ITF, ITF2357; ALT, alanine aminotransferase.

ptosis in malignant liver-derived cell types, including a differential regulation of pro- and antiapoptotic cellular proteins in malignant and nonmalignant cell types.[10,28,29] Our present study demonstrates for the first time that the HDAC-I compounds VPA and ITF2357 are able to mediate a complex transcriptional "reprogramming" by histone hyperacetylation leading to a specific priming of human hepatoma cells to TRAIL-induced apoptosis. Similar to earlier studies,[18,24,25] we observed highly resistant human hepatoma cells exhibiting only close-to-baseline sub2n DNA levels after single TRAIL incubation, but we achieved pronounced apoptosis induction following cotreatment with HDAC-I compounds and TRAIL. Furthermore, we found that two factors are essential for hepatoma cell sensitization to TRAIL: (1) a 24-hour single HDAC-I preincubation period and (2) ongoing HDAC inhibition (via HDAC-I treatment) covering the whole period of TRAIL exposure (data not shown).

Although several studies tried to address mechanisms accounting for TRAIL susceptibility of cancer cells, the key determinants still remain controversial. Despite the initial idea of altered death receptor expression being responsible for an inadequate TRAIL response,[30] there is growing evidence that dysregulated intracellular signaling pathways may be more important to the development of TRAIL resistance,[6] particularly in the case of TRAIL-resistant hepatoma cells. Studies that analyzed TRAIL-resistant human hepatoma cells in comparison with healthy tissue-derived nonmalignant cells confirmed similar TRAIL-R1 and -R2 distribution levels for both the malignant and nonmalignant cell entities.[23] Thus, the argument of low receptor expression causing insufficient TRAIL response has to be regarded as rather improbable.

However, in the special context of carcinoma cell priming to TRAIL-induced apoptosis, a recent report proposed a correlation between upregulated TRAIL-R1 and -R2 expression levels and restored TRAIL sensitivity due to HDAC-I treatment in leukemic cell lines.[31] In contrast, our hepatoma cell study revealed no alteration or even decreased death receptor presentation on the cellular surface in the course of ITF2357 or VPA treatment (Fig. 2), but a remarkable induction of apoptosis by TRAIL even for cells showing a downregulation in death receptor expression levels. These data are consistent with previous work showing that some hepatoma cells can be sensitized to TRAIL by a pretreatment with cisplatin, which did not lead to a concurrent alteration of the amount of membrane-bound TRAIL receptors.[32] Consequently, these results strongly imply that in hepatoma-derived cell types, mechanisms other than a deregulation of death receptors are responsible for HDAC-I-mediated sensitization to TRAIL.

Searching for other major contributors capable of disturbing TRAIL-promoted proapoptotic signaling pathways in cancer, the intracellular expression level of FLIP is regarded as an interesting candidate. This antiapoptotic protein, which is a potent inhibitor of TRAIL-mediated cell death by preventing caspase-8 activation,[5,33-36] is constitutively expressed in human hepatoma cells but is almost undetectable in untransformed human hepatocytes.[16] Concerning HDAC-I-mediated TRAIL sensitization, some compounds that are capable of reducing intracellular FLIP levels were found to facilitate apoptosis in TRAIL-resistant colon carcinoma cells[37] as well as in leukemic cell lines.[31] In accordance with these results, we found evidence that TRAIL-induced hepatoma cell death in the course of VPA or ITF2357 treatment is associated with a remarkable downregulation of FLIP mRNA transcript and protein levels (Fig. 4A-B). Interestingly, FLIP antisense oligonucleotides also have been reported as a tool for priming TRAIL-resistant human hepatoma cells susceptible to death ligand-mediated apoptosis.[16] Taken together, our data suggest that the reduction of FLIP expression levels achieved by HDAC-I intervention might play a decisive role in HDAC-I-promoted TRAIL sensitization enabling restoration of dysregulated extrinsic apoptotic pathways that account for the inherent TRAIL resistance of hepatoma cells.

Recently, c-myc has been identified as a determinant of TRAIL sensitivity by showing that overexpression of c-myc is able to induce a decrease in cellular FLIP levels.[38] At first glance, this observation might be contradictory to our results, because some HDAC-I substances have been found to downregulate c-myc expression.[39,40] However, looking at our own microarray experiments with HepG2 cells, we found a significant upregulation of c-myc in the course of VPA or ITF2357 treatment (data not shown). It is tempting to speculate that a complex regulation pattern after HDAC inhibition mediates apoptosis via upregulation of c-myc, which has been found to be suppressed in hepatoma cells.[41,42] This complex regulation pattern in hepatoma cells is currently addressed in a separate study.

In contrast to our investigations looking at TRAIL-resistant hepatoma-derived cell types, two recent reports convincingly demonstrated that in TRAIL-sensitive leukemia-derived cells TRAIL itself is upregulated by HDAC-I incubation, being responsible for an efficient induction of tumor cell apoptosis.[14,15]

Furthermore, it was supposed that TRAIL induction might be the key event for a cancer-selective apoptogenic activity of HDAC-I compounds.[15] This assumption may be true for TRAIL-sensitive tumor cell types, but according to our data, it cannot be regarded as the explanation for HDAC-I-mediated apoptosis in TRAIL-resistant cell types (such as hepatoma cells). Therefore, our data add important information by showing that TRAIL-resistant hepatoma cells can be primed to TRAIL-induced cell death, resulting from a HDAC-I-induced modulation of intracellular apoptosis modifying protein levels.

Each and every new therapeutic strategy employing TRAIL with a potential *in vivo* application has to look carefully for unwanted and toxic side effects. Surprisingly, when undertaking our combinatorial HDAC-I/TRAIL approach, we found a highly differential regulation pattern in human hepatoma cells (downregulation of cellular FLIP transcript and protein levels) versus PHH (upregulation of cellular FLIP transcript and protein levels). The impact of this observation is at least twofold: on the one hand, a combinatorial HDAC-I/TRAIL treatment seems to be a potent approach to destroy human hepatoma cells; on the other hand, an upregulation of FLIP in nonmalignant, healthy human hepatocytes achieved by preincubation with HDAC-I compounds even further protects these cells from potential toxic side effects of TRAIL. In addition to this, the applicability of our combinatorial HDAC-I/TRAIL approach to future preclinical or even clinical settings is simplified by the fact that VPA has a well-proven clinical safety profile and that ITF2357 has already found its way into clinical trials in a different context.

In conclusion, we have characterized the ability of two HDAC-I substances, VPA and ITF2357, to overcome the inherent resistance of human hepatoma cells toward TRAIL. Moreover, we found a differential regulation pattern of the apoptosis-modifying cellular protein FLIP in malignant and nonmalignant cell types that might contribute to the differential induction of cell death in the course of combinatorial HDAC-I/TRAIL applications. The upregulation of FLIP within nonmalignant liver cells may even enhance the therapeutic window for an application of death-inducing ligands *in vivo*, which might open up a new and highly specific treatment option for patients with advanced HCC.

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