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Proteasome inhibition overcomes TRAIL resistance in human hepatoblastoma cells

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1. ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is responsible for cell death in many cancer cells while being non-toxic for most normal cells. In this study, we investigated the role of TRAIL in human hepatoblastoma (HB) cells and analyzed different approaches to reverse TRAIL resistance in these tumors. Death receptors DR4 and DR5 expression was found on all analyzed primary HB samples and on the cell lines HuH6 and HepT1 by immunofluorescence staining. Recombinant TRAIL alone did not induce *in vitro* cytotoxicity. Decoy receptor blocking by antibodies led to moderate effects in HepT1 but not in HUH6 cells, whereas FLIP knock-down using siRNA rendered HUH6 cells but not HepT1 cells sensible to TRAIL. Bcl-2 inhibition with ABT-737 enhanced TRAIL-mediated apoptosis in all HB cells. Strongest cytotoxic TRAIL effects were seen in HB cell lines with synchronous proteasome inhibition using bortezomib. FLIP and Bcl-2 contributed to the TRAIL resistance in HB. Overcoming TRAIL resistance in HB by proteasome inhibitors has been identified a possible additive to improve treatment results in HB patients with drug resistant tumors.

2. BACKGROUND

Despite good treatment results of children with low stage or low risk human hepatoblastoma (HB), there still are subgroups of this entity associated with a poor prognosis. This especially includes advanced HB (relapsed, multifocal, and metastasized tumors), in which chemotherapy resistance plays an important role resulting in reduced resection rates, increased numbers of recurrence and tumor progression. Analysis of treatment results through various collaborative multicenter trials demonstrated the need for additional or alternative treatment options in order to improve the prognosis of affected children (1,2,3). An increasing number of reports described a linkage of anti-tumor therapy to different cell death mechanisms such as apoptosis, mitotic catastrophe, necrosis, autophagy as well as a permanent cell arrest with phenotype characteristics of senescence (4). Apoptosis is an important factor in anticancer treatment. Anti-apoptotic mechanisms have been observed in various malignancies contributing to a decreased efficacy of chemotherapy. Modulating apoptosis is therefore an emerging field for developing new anti-cancer drugs.

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) is part of a natural mechanism to kill tumor cells by the immune system (5). Normal cells express neutralizing decoy receptors (DcR1 and DcR2) and are not sensitive to TRAIL. Binding of TRAIL to its death receptors (DR4 and DR5) in tumor cells activates the extrinsic apoptotic pathway by recruiting procaspase 8 into the death-inducing silencing complex. Cleavage of the BH-3 only peptide Bid by caspase 8 links the apoptotic TRAIL signal to the mitochondrial pathway and the subsequent release of cytochrome c. Progression into the apoptotic pathways is counteracted by anti-apoptotic proteins such as Bcl-2, FLIP and XIAP. FLIP has been identified as an independent adverse prognostic factor in various cancer types (colon, endometrial, Burkitt's lymphoma and ovary) (6,7). Bcl-2 proteins are overexpressed in many cancers and are believed to contribute to tumor initiation, progression and resistance to therapy (8).

TRAIL is a promising candidate for the treatment of cancer because it triggers apoptosis in tumor cells while having no such effect in normal cells. TRAIL has been demonstrated to be an effective anti cancer agent in various malignancies both, in preclinical and clinical settings (9). TRAIL-based approaches to cancer therapy include i) the systemic administration of recombinant, soluble TRAIL protein with or without the combination of traditional chemotherapy, ii) novel anti-cancer agents to agonistic monoclonal antibodies directed against functional TRAIL receptors, and iii) TRAIL gene transfer therapy. However, the phenomenon of TRAIL resistance has been described for some tumors mediated through various factors along the apoptosis pathway (10,11). Especially liver tumors have been reported to display a variety of TRAIL resistance mechanisms, consecutively, a main focus of analyses lies on restoring sensitivity of tumors cells to TRAIL-mediated cytotoxicity (12-14). Recently, a successful combined treatment approach using TRAIL and histone deacetylase inhibitors has been reported for HB and pediatric HCC cells (15,16). However, the exact role of TRAIL and related resistance in HB has so far not been clarified systematically. In our study we evaluated the eligibility of TRAIL as alternative treatment option in HB *in vitro*.

3. METHODS

3.1. Tissue samples

We analysed tissue samples from 8 patients undergoing liver resection for HB at our department between April 2002 and August 2005. Mean age of the patients at surgery was 26.7 months (range 5-61.5). Patients were either treated according to the protocol HB99 of the GPOH, or according to the SIOPEL 2 protocol (Table1). Serum alpha-fetoprotein levels were above 100 ng/ml in all cases. Patients received Ifosphamid/Cisplatin/Adriamycin (IPA), Cisplatin/Doxorubicin (Plado), or Carboplatin/Etoposid (VP16), respectively. Three patients received high dose Carboplatin and VP16 chemotherapy with subsequent stem cell rescue (HD-SCR). Immediately after resection, the tumor samples were shock frozen and stored in liquid nitrogen until use. The study was done according to the ethical guidelines of the 1975 Declaration of Helsinki and informed consent was obtained from the parents of all patients before operation.

3.2. Cell lines and culture conditions

The HB cell lines HepT1 and HUH6 were used for all experiments. HepT1 cells were derived from a multifocal embryonal HB (17). HUH6 cells were derived from a mixed HB (18). Tumor cells were grown in DMEM (GIBCO BRL, Carlsbad, CA) supplemented with 10% FCS. Cell cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37°C.

3.3. Viability assay

HB cells (10.000 cells/100µl) were cultured in 96-well plates. Recombinant SuperTRAIL (Alexis Biochemicals, Lörrach, Germany) was added at concentrations of 5-50 ng/ml. For blocking experiments cells were incubated with polyclonal antibodies against decoy receptors DcR1 and DcR2 (Abcam, Cambridge, UK) each at 1 µg/ml 30 minutes prior to TRAIL incubation for 48h. All assays were performed 3 times in triplicates. Cell viability was assessed using the MTT-assay (EZ4U, Biomedica, Wien, Austria). Growth inhibition was calculated by normalization between background of cultures without cells and untreated cultures as control experiments. Viability was also analyzed after incubation with bortezomib (Janssen Cilag, Neuss, Germany) and after antagonization of Bcl-2 using ABT-737 (Abbott Laboratories Inc, IL) concomitant with SupperTRAIL.

3.4. Transfection with siRNA against FLIP

Knockdown of FLIP was performed with predesigned siRNA HSCFLAR-4 and -5 combined with HiPerFect transfection reagent at 5 nM siRNA and 3 µl transfection reagent for 37 ng siRNA (all from Qiagen, Hilden, Germany). Specific knock-down of XIAP was done with siRNA from Cell Signaling Tech. (Danvers, MA). Three days after transfection cells were plated for proliferation assays, for immunofluorescence staining, and Western Blot (WB) (19) to assess knock-down efficiency.

3.5. Immunofluorescence

Immunofluorescence was used to detect TRAIL receptor expression (DR4 and DR5) on HB cells as well as FLIP (Abcam) and XIAP (Cell Signaling) expression. $1x10^4$ cells were used in 500µl medium and seeded out on culture slides (BD Biosciences, Heidelberg, Germany). Fixation was done in formaldehyde 4.5% followed by blocking with goat serum 1.5% (Jackson ImmunoResearch, Suffolk, UK) for 30 minutes. Incubation with primary antibody took place for 2 hours at room temperature. Secondary antibody FITC-conjugated Goat anti-mouse IgG (1:100, Jackson ImmunoResearch) and DAPI (1:10000, Sigma, Munich, Germany) were used for 30 minutes. Immunofluorescence was carried out on a Zeiss Axio Scope epifluorescence microscope (Carl Zeiss, Oberkochen, Germany) with a MRC5 camera. Images were processed using AxioVision software.

3.6. Detection of apoptosis

Apoptosis was monitored using the specific cleavage of the fluorogenic DNA-binding dye from a

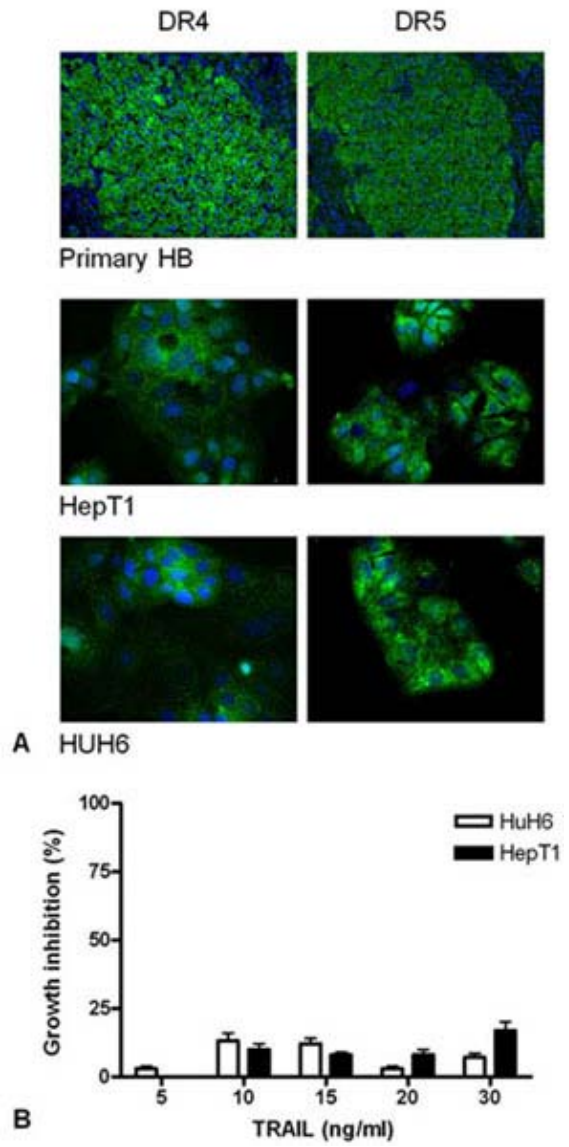

Figure 1. Expression of dead receptors on human hepatoblastoma tissue and cell lines. A) Immunofluorescence analysis revealed high expression of DR4 and DR5 as green fluorescence on primary HB, HepT1 and HuH6 cells. Blue fluorescence of DAPI denotes nuclear staining. B) HB cells HuH6 and HepT1 were cultured with recombinant TRAIL. Growth inhibition was determined in a cell proliferation assay and related to untreated cell cultures. Ascending concentrations of TRAIL did not result in growth inhibition in either of the cell lines.

peptide by activated caspase-3. For live cell caspase-3 detection, confluent hepatoblastoma cells were treated on 96 well plates with bortezomib and TRAIL for 24 h. DEVD-NucView488 (Biotrend, Cologne, Germany) was added at 5 µM and cells were fixed with 3.75% formaldehyde 90 min later followed by staining with 0.1 µg/ml Hoechst33342 for microscopic analysis. In some experiments, cells were trypsinated and analyzed by flow cytometry using the FACScalibur cytometer (Becton Dickinson, Heidelberg, Germany) and CellQuest software.

3.7. Cytotoxicity assay

Peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated from EDTA blood samples with Biocoll separating solution (Biochrom, Berlin, Germany) by density gradient centrifugation. Isolated PBMCs were incubated at 37 °C for up to 18 h in the presence of 200 U/ml IL-2 (Chiron GmbH, Ratingen, Germany). HB cells were labeled with BATDA (Perkin-Elmer, Rodgau, Germany) as described previously (20). Stained cells (5000/well) were incubated with anti-TRAIL (clone RIK-2, eBioscience, Frankfurt, Germany) at 10 µg/ml. PBMCs were added as effectors at a previously determined optimal ratio of 10:1 (E:T). Controls for spontaneous release include only target cells. Maximal lysis was induced in controls with 1% Triton X100 (Sigma-Aldrich, Munich, Germany). Target lysis was monitored by fluorescence 4 h later using europium solution (Perkin-Elmer, Rodgau, Germany). The percentage of specific cytolysis was calculated using the formula: ((experimental counts/minute – spontaneous counts/minute) / (maximal counts/minute – spontaneous counts/minute)) x 100.

3.8. Statistical analyses

Statistical analysis of treatment effects were carried out by non-linear regression using the software JMP IN 5.1 (SAS Institute, Cary, NC).

4. RESULTS

4.1. Expression of TRAIL receptors on hepatoblastoma cells

To assess the susceptibility to TRAIL, we analyzed TRAIL death receptor expression (DR4 and DR5) on primary HB samples and on HB cell lines using immunofluorescence. There was strong expression of both receptors on all analyzed samples suggesting a relevant binding potential of HB cells for TRAIL (Figure 1A). DR4 and DR5 expression on primary samples was detectable regardless of the clinical stage, previously applied chemotherapy, and histological subtype of the tumors (Table 1). Cell lines expressed more DR5 than DR4. The DR5 receptor is known to be the major receptor involved in TRAIL-induced apoptosis.

4.2. Effects of TRAIL incubation on hepatoblastoma cells

To analyze the effects of TRAIL on HB cells we cultured the cells in media containing ascending concentrations of TRAIL. There was no decrease of the viability of HB cells supplemented with TRAIL even at 30 ng/ml (Figure 1B). To investigate the cause for resistance to TRAIL, we analyzed blocking of TRAIL receptors without death domain (DcR1 and DcR2) simultaneously to TRAIL incubation. Blocking of DcR1 and DcR2 did not significantly alter the cytotoxic effects of TRAIL on HUH6 cells suggesting that there is a different mechanism responsible for TRAIL resistance in this cell line. In HepT1 cells, there was a mild cytotoxic effect of DcR-mAb (19±5% growth inhibition), which was further increased by

Table 1. Expression of TRAIL receptors DR4 and DR5 in hepatoblastoma tissue

Sample No.	Pretext	Histology	Chemotherapy	DR4	DR5
1	I	Mixed	IPA	++	+++
2	II	Mixed	Plado	+	+
3	II	Mixed	Plado	+++	++
4	III	Epith. (fetal)	Plado	+	+
5	III	Epith. (fetal)	HD+SCR	+	+
6	III	Epith. (fetal)	Carbo/VP16	++	++
7	IV	Epith. (fetal+embryonal)	HD+SCR	+	+++
8	IV	Epith. (fetal+embryonal)	HD+SCR	+	+++

+ low, ++ moderate, +++ high expression, Pretext classification of patients according to SIOPEL, Mixed histology: Epithelial and mesenchymal compounds, Chemotherapy: Ifosphamid/Cisplatin/Adriamycin (IPA), Cisplatin/Doxorubicin (Plado), Carboplatin/Etoposid (Carbo/VP16), high dose Carboplatin and VP16 and stem cell rescue (HD-SCR)

co-incubation with TRAIL (33±6%). These findings contain evidence that decoy receptors at least partially contribute to TRAIL resistance in HepT1 cells.

4.3. Effects of FLIP inhibition on hepatoblastoma cells

In order to investigate further possible mechanisms of TRAIL resistance in HB, we evaluated the role of the flice-like inhibitory protein (FLIP) on the apoptotic pathway in the tumor cells. FLIP is a homologue of caspase-8 that is recruited to the death-inducing signaling complex, thereby inhibiting the activation and cleavage of caspase-8 and the inhibitor of apoptosis proteins (IAP, e.g., XIAP, survivin, and cIAP), suppressing apoptosis by inhibition of caspases 3, 7, and/or 9 (21-24). FLIP is therefore a potent inhibitor of human death receptor induced apoptosis. Immunohistochemistry revealed strong expression of FLIP (L), the long form of FLIP, on both HB cell lines (Figure 2A). Next we examined whether there was also a functional role of FLIP in the tumor cells. Therefore HB cells were transfected with FLIP-siRNA for a transient FLIP down regulation. Western blot analysis revealed a potent down regulation of FLIP through the two siRNA used (Figure 2B). Incubation of transfected HB cells with TRAIL had no cytotoxic effects in HepT1 cells. However, both tested siRNA significantly sensitized HUH6 cells to TRAIL (Figure 2B). These findings indicate differential resistance mechanisms in the two cell lines and suggest a possible role of FLIP (L) in modulating TRAIL-related apoptosis in HUH6 cells.

4.4. Inhibition of Bcl-2 does not induce sensitivity of hepatoblastoma cells to TRAIL

Over expression of anti-apoptotic Bcl-2 family members has been associated with chemotherapy resistance in various human cancers (25), and a positive effect of Bcl-2 knock-down on chemotherapy of HB has been previously reported by our group (26). However, a possible association with the TRAIL pathway has not been investigated so far. HB cells were incubated with ABT-737, a small-molecule BH3 mimetic that binds to and antagonizes Bcl-2. ABT-737 synergistically enhances TRAIL-mediated cytotoxicity in various human cancer cell lines (27,28). In both HB cell lines, ABT-737 induced dose dependent apoptosis as quantified by flow cytometry of the cells with activated caspase-3. In order to evaluate possible synergistic effects, we next co-incubated tumor cells with TRAIL and ABT-737 at increasing concentrations. A significant increase in apoptotic HuH6 cells could be observed (Figure 3A, F-Test $p<0.05$). However, there was no decrease of cell vitality using this combined treatment regime in HepT1 cultures (Figure 3B).

4.5. Proteasome inhibition overcomes TRAIL resistance in hepatoblastoma cells

It has been demonstrated in various cells that proteasome inhibition can lead to a shift in the balance of pro- and anti-apoptotic factors resulting in profound apoptosis or sensitization of hepatoma cells to the pro-apoptotic cytokine TRAIL (29). Pre-treatment with 5ng/ml Bortezomib for 24 h caused a marked increase of apoptotic hepatoblastoma cell numbers (Figure 4A). This could be enhanced by co-treatment with TRAIL. There was a significantly higher effect of combined Bortezomib (5 and 10 ng/ml) and TRAIL (50 ng/ml) treatment compared to treatment with TRAIL alone or control cultures ($p<0.05$, two way ANOVA, Figure 4B). Besides a direct anti-proliferative effect of Bortezomib on HB cells, proteasome inhibition specifically induces expression of ligands for immune cells, leading to a specific stimulation of blood cells with anti-tumor activity. To investigate whether Bortezomib treatment of hepatoblastoma cell lines also influenced cytotoxicity of peripheral blood monocytes (PBMC), we cultured PBMCs in the presence of untreated and Bortezomib-treated hepatoblastoma cells. Specific lysis of hepatoblastoma cells increased with the concentration of Bortezomib (Figure 4C). At a ratio of 10 PBMC to one HB cell a significant tumor cell killing was promoted by 4 ng/ml Bortezomib. This could be partially inhibited by blocking TRAIL. Low-dose Bortezomib thus mediated a dual anti-tumor effect combining inhibition of tumor cell proliferation with a specific priming of malignant cells for immune cell recognition.

5. DISCUSSION

In this study, we provide evidence that various substances are able to increase the apoptotic sensitivity of HB cells to receptor mediated apoptosis. TRAIL is critically involved in tumor rejection through natural killer (NK) cell-mediated immune surveillance. Clinical trials have been initiated in cancer patients using soluble recombinant TRAIL and agonistic monoclonal antibodies targeting TRAIL-receptors (30). These drugs were generally well tolerated by patients, however, the percentage of patients with adequate tumor response was low. TRAIL and agonistic anti-TRAIL-R therapies thus appear limited to patients with TRAIL-sensitive tumors. It has been suggested that the efficacy of TRAIL targeting therapies could be improved by the availability of diagnostic methods determining TRAIL sensitivity of clinically detectable human cancers (31). We showed in a small patient's cohort, that HB tissue expresses at least one

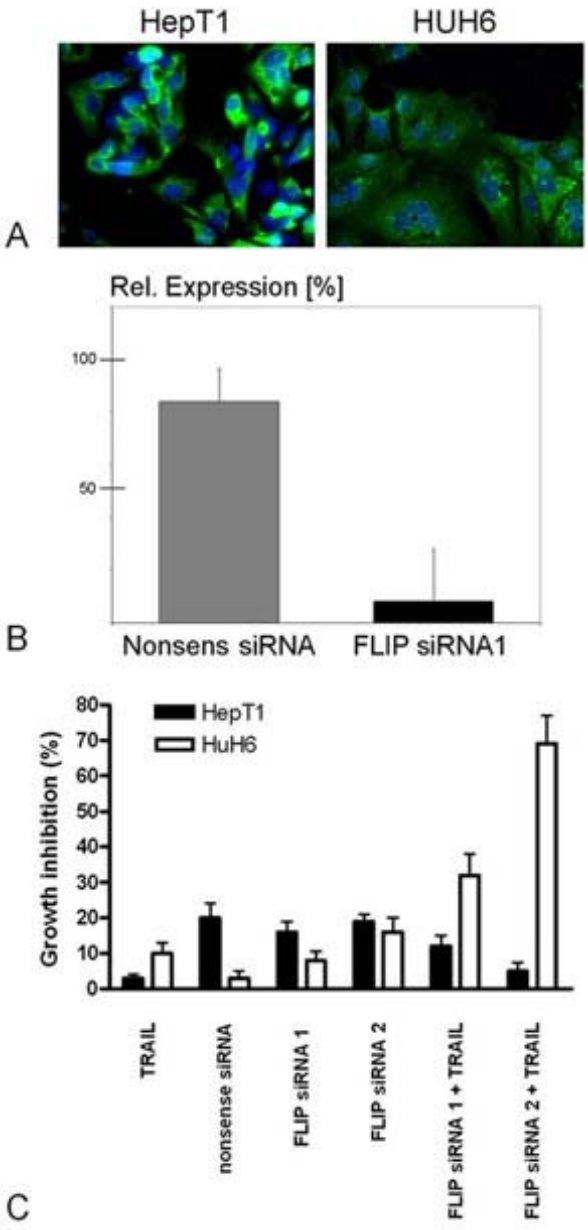

Figure 2. Influence of FLIP on TRAIL sensitivity in HB cells. A) Expression of FLIP in HB cells was detected by immunofluorescence as a bright green staining. B) HB cells were transfected with siRNA and FLIP protein was quantified by WB and densitometry 48 h later. Relative expression was calculated to untreated cells. FLIP-siRNA led to a significant downregulation of FLIP in HB cells. C) HB cells were transfected with two FLIP-specific siRNA and a nonsense siRNA. Growth inhibition was measured in a proliferation assay with 30 ng/ml TRAIL for 48h. Data represent mean and SD of triplicate experiment. HuH6 cells showed significant enhanced growth inhibition through FILP knock-down whereas there were no changes in HepT1 cells.

death receptor for TRAIL independent from clinical or biological characteristics. The regular expression of TRAIL death receptors in HB would suggest a general sensitivity of HB to TRAIL. However, in a preclinical model for HB, we found an impaired apoptosis inducing effect of TRAIL on HB cells which is in accordance with observations in breast and colon cancer cells. The low response of tumor cells to TRAIL may facilitate the escape from the control through the innate immune system, this may lead to tumor dissemination even in tissues with high contents of macrophages and natural killer cells such as the liver, lung and bone marrow. Trials are still ongoing, especially involving combination of TRAIL agonistic agents with current chemotherapy drugs (9). The aim of our study was to sensitize HB cells to TRAIL induced apoptosis through modulating key proteins in the apoptotic pathway. First we

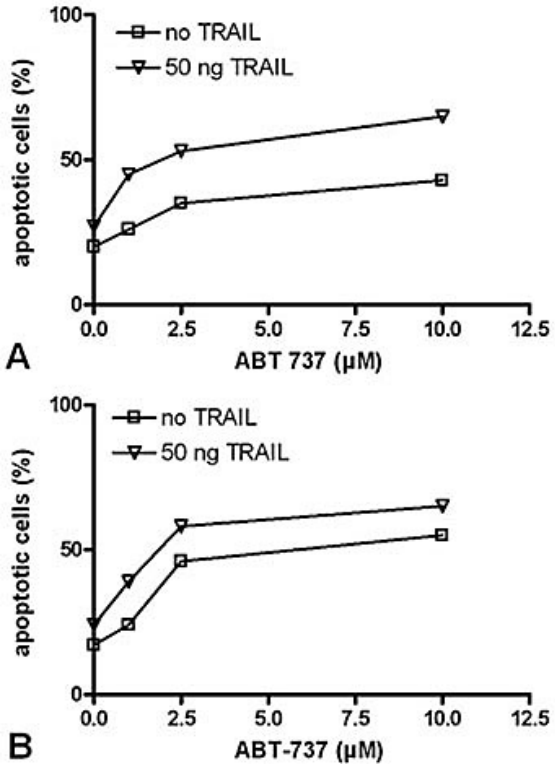

Figure 3. Apoptosis induction in HB cells with TRAIL in combination with the bcl-2 inhibitor ABT737. HB cells were cultured with 50 ng/ml TRAIL and increased concentration of ABT-737 at indicated concentrations for 48 h. Apoptotic cells were detected by Caspase 3 activation and flow cytometry. Data represent percentage of apoptotic cells from total cells in the culture. There was an increase of apoptotic cells through combined treatment, however, differences were not significant.

inhibited key anti-apoptotic proteins such as FLIP, XIAP, and Bcl-2. However, a broad reprogramming of the tumor cells with proteasome inhibitors was necessary to sensitize HB cells to TRAIL induced apoptosis.

Over-expression of anti-apoptotic Bcl-2 family members has been associated with chemotherapy resistance in various human cancers, and preclinical studies have shown an activity of agents targeting anti-apoptotic Bcl-2 family members as single or combined approach (32). Clinical trials of several investigational drugs targeting the Bcl-2 family (oblimersen sodium, AT-101, ABT-263, GX15-070) are ongoing (32). In HB cells, siRNA knock-down of Bcl-2 improved the chemosensitivity of HuH6 and HepT1 cell to Cisplatin, Etoposide, Doxorubicin and Taxol (26). Using ABT-737 for inhibition of the interaction between Bcl-2 and Bax, TRAIL-mediated apoptosis could be enhanced only in one of the tested HB cell lines. This suggests a diverse interference on the level of the apoptotic pathways in histologically differing HB cell lines. The different biological behavior of HB has been previously observed in clinical studies as well as in experimental settings (33).

It has been reported that a mitochondrial block and expression of XIAP leads to resistance against TRAIL-induced apoptosis during progression of dissemination of a colorectal carcinoma (16,34). Although XIAP was detected in HB cells at high levels and a knock down of XIAP was gained, the TRAIL-mediated apoptosis in HB cells was not enhanced. This suggests a block of the apoptosis pathways proximally to the TRAIL-receptor. FLIP inhibits recruiting of Caspase 8 to the DR4 and DR5 receptors and serves as potential target to facilitate apoptosis. In hepatoma cells FLIP was detected as a main factor mediating apoptosis resistance (35). Knock-down of FLIP in HB cells successfully restored TRAIL sensitivity in HuH6 cells, which were also sensitized by Bcl-2 inhibition.

Overcoming resistance to TRAIL by modulation of a single molecule in the apoptotic pathway (Bcl-2, FLIP, or XIAP) using BH3-mimicry and siRNA did not have the expected effects in all analyzed HB cells. This might be caused by expression of other anti-apoptotic proteins such as cIAP-1, Survivin, and heat shock proteins, which have been observed in HB cells (36). The heterogenic response to punctual intervention at only one site in the apoptotic pathway of HB cells revealed a tissue type dependency of the anti-apoptotic mechanism in HB cells. A broad reprogramming of the tumor cells is mediated by histone deacetylase inhibitors. One member of this inhibitors, SAHA, stimulated apoptosis in both hepatoblastoma derived cells HepG2 and HuH6 cells but not in primary human hepatocytes (15,16,37). Therefore the therapeutic window for clinical applications of TRAIL, as a highly specific new treatment option for advanced solid tumors, will presumably not face adverse toxicity.

Proteasome inhibitors promoted sensitization of HB cells to receptor mediated apoptosis, which was broad and independent from the histological subtype. The clinically used proteasome inhibitor bortezomib increased the accumulation of JNK-1 and AP-1 with activation of caspase-8 in a variety of tumor cells (37). Changes related to members of the Bcl-2 family were predominant with increased levels of pro-apoptotic members and decrease levels of anti-apoptotic proteins that facilitates cell death (37). Bortezomib has been shown to enhance the activity of commonly used drugs in drug resistance models for cancer (38). However, bortezomib has also an inhibitory effect on NK cells, which are a main source of TRAIL (39). Therefore a mono-therapy with bortezomib will most likely be less effective than a combined approach including drugs activating TRAIL-receptors.

Commonly used drugs in treatment protocols of HB act as alkylating agents (Cisplatin), disturb RNA-synthesis (Doxorubicin), inhibit organization of microtubules (Paclitaxel), or interfere with DNA-repairing mechanisms (Etoposid), they all activate apoptosis. Sensitizing HB cells to apoptosis could possibly lead to dose reduction of established drugs, might prevent development of multi drug resistance and might reduce side effects such as cardiomyopathy. Among patients with drug resistant breast cancer, some had evidence of a clinical

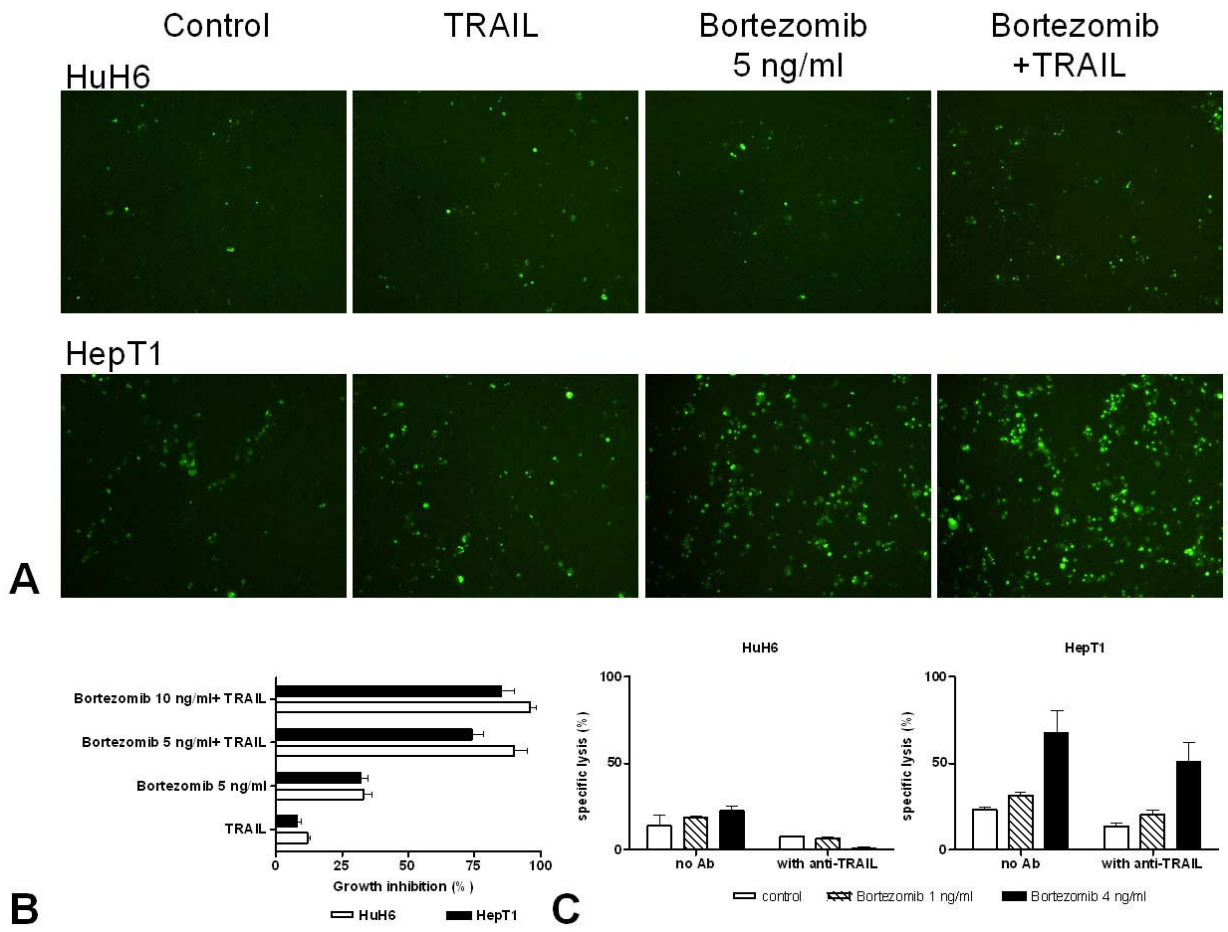

Figure 4. Effect of Bortezomib on TRAIL mediated apoptosis in HB cells. HuH6 and HepT1 were incubated with 1ng/ml Bortezomib and 50 ng/ml TRAIL. (A) Apoptosis was detected with the Caspase 3 substrate NucView 488. Green fluorescence denote apoptotic cells with activated Caspase 3 in a 24 h assay. (B) Growth inhibition was monitored in a 72 h assay. Combined inhibition with Bortezomib and TRAIL leaded to a significant growth inhibition in both HB cell lines. (C) HB cells were cultured with Bortezomib for 48 h previous analysis of tumor cell lysis with PBMC. In some experiments endogenous expressed TRAIL was blocked by 10 µg/ml antibody to TRAIL. Bortezomib treatment increased hepatoblastoma cell lysis and was mediated in part through TRAIL.

benefit of combining the proteasome inhibitor bortezomib with anthracyclines (40). Bortezomib also showed a pharmacologic advantage increasing platinum accumulation in cancer cells and enhanced cell killing with CDDP in a synergistic manner, suggesting a possible combination in treatment of relapsing HB (41).

Taken together, a tissue type independent and broad sensitization of HB cells to receptor mediated apoptosis (e.g.TRAIL) could be achieved with proteasome inhibitors. Proteasome inhibitors may be considered as a therapeutic option for treatment refractory HB.

6. ACKNOWLEDGEMENTS

Written consent for publication was obtained from all patients' parents.

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