Betulinic acid treatment promotes apoptosis in hepatoblastoma cells

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Abstract. Hepatoblastoma (HB) represents the most common malignant liver tumor in children with a dismal prognosis for patients with advanced disease. This study provides evidence that the naturally occurring pentacyclic triterpenoid betulinic acid (BA) is highly effective against HB. We demonstrate that BA has a strong cytotoxic effect on HB cells in a dose-dependent manner by impinging on cell viability and causing massive induction of programmed cell death. Apoptotic features including morphological changes, membrane asymmetry and proteolytic cleavage of caspase 3 and poly(ADP-ribose) polymerase were frequently found in BA-treated HB cells, which is suggestive of the mitochondrial intrinsic apoptotic pathway. In contrast, the hepatocellular carcinoma (HCC) cell line HepG2 was resistant to BA treatment. This insensitivity was dependent on the high expression of survival factors, such as *Survivin* and *BCL2*. Interestingly, BA treatment led to a significant decrease in expression of the hedgehog target genes *GLI1*, *PTCH1* and *IGF2* in HepT3 cells. In conclusion, we demonstrate that BA is capable of inducing apoptosis in HB cells and thereby might be a hopeful new strategy for treating HB, especially those with an activated hedgehog signaling pathway.

Introduction

Hepatoblastoma (HB) is the most common hepatic tumor in childhood with an incidence of ~1 per million children below 15 years of age (1). This tumor especially affects young children between 6 months and 3 years of age with an overall median age of 18 months at diagnosis (2,3). Histologically, there are two major subtypes of HB; epithelial and mixed epithelial/mesenchymal. The majority of HB are epithelial tumors showing cells with fetal and/or embryonal hepatic differentiation, whereas mixed HB also contain mesenchymal elements such as immature fibrous tissue or spindle cells and osteoid (3). Although HB treatment has dramatically improved during the past 20 years by combining chemotherapy regimens with surgery, the fatal outcome of high-risk patients with advanced or recurrent HB makes new treatment strategies essential (4,5).

On the molecular level, a high proportion of HB exhibits loss of heterozygosity and/or imprinting defects at the chromosomal band 11p15.5, which harbors the insulin-like growth factor-II (*IGF2*) gene (6-8). It is commonly accepted that overexpression of *IGF2* is one of the driving forces in the development and progression of HB (9). Several lines of evidence indicate that IGF2 promotes cell survival and inhibits apoptosis via the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway, which is frequently altered in cancer (10,11). The serine/threonine kinase AKT is known to elicit its effects through phosphorylation and inactivation of proapoptotic factors and it has been suggested that this may afford tumor cells a survival advantage (12). Therefore, identifying new drugs that inhibit PI3K/AKT signaling and induce apoptosis might be a promising strategy to develop more effective therapies for HB in the future.

The plant-derived betulinic acid (BA) has been reported to exhibit anti-tumoral activity in a variety of human cancers such as melanoma (13) and tumors of the lung, colon, prostate and ovary (14), whereas BA showed minimal toxicity against normal cells (15). BA is known to induce apoptosis by the intrinsic apoptosis pathway followed by the activation of specific caspases (16-18). Moreover, BA showed a beneficial effect in drug-resistant tumor cell lines (19). Since other embryonal tumors such as medulloblastoma and neuroblastoma have been described to display PI3K/AKT-associated features of cell survival (20,21), but could be successfully treated with BA (22,23), we wondered whether BA has also a cytotoxic effect on HB.

Here, we report that BA treatment leads to a dose-dependent growth inhibition and induction of apoptosis in HB cells. In contrast, the hepatocellular carcinoma (HCC) cell line HepG2 displayed no evidence for apoptosis, which could be explained by an increased expression of survival genes such as *Survivin* and *BCL2* in this cell line. Furthermore, we demonstrated that the expression of the hedgehog target genes *GLI1*, *PTCH1* and *IGF2* was significantly decreased in

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hedgehog-activated HB cells upon BA treatment. These findings suggest that BA is able to inhibit tumor growth of HB *in vitro* and that it may be a hopeful new strategy for the treatment of this malignant liver tumor.

Materials and methods

Tumor cell lines. The three human HB cell lines HUH6 (Japanese Collection of Research Bioresources, Osaka, Japan), HepT1 (24) and HepT3 (6), as well as the HCC cell line HepG2 (ATCC, Manassas, VA, USA) were used for all experiments. Cell lines were maintained as the suppliers recommended. Human fibroblasts were grown in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin.

Western blot analysis. Protein (20 μg) was isolated and separated on 12% SDS-PAGE under reducing conditions as described earlier (25). Proteins were then transferred to nitrocellulose membranes (GE Healthcare, Piscataway, NJ, USA). The membranes were incubated with PBS containing 0.1% Tween-20 and 5% non-fat dry milk to block non-specific binding, then incubated with rabbit anti-human AKT, rabbit anti-human phospho-AKT (Ser473), mouse anti-human caspase 3, rabbit anti-human cleaved caspase 3, rabbit anti-human poly(ADP-ribose) polymerase or rabbit anti-human ß-actin (all from Cell Signaling Technology, Danvers, MA, USA) for 1.5 h and thereafter for 1 h with the appropriate horseradish peroxidase-conjugated secondary antibody (DakoCytomation, Hamburg, Germany). Signals were visualized using the ECL chemiluminescence detection system (GE Healthcare).

Cell viability and morphology. Cell growth was assessed using the Cell Proliferation Kit I (Roche Diagnostics, Penzberg, Germany) according to the manufacturer's protocol. Cells were seeded at a density of $5x10^3$ cells/96-well plate (Nunc, Wiesbaden, Germany) and after overnight attachment treated for 24 h with 0-50 μg/ml BA (BioSolutions, Halle, Germany) dissolved in DMSO. Cell viability was measured after addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) labeling reagent on the GENios reader (Tecan, Männedorf, Switzerland) at a wavelength of 595 nm. Morphological changes of incubated cells were documented with a Zeiss inverted phase-contrast microscope equipped with a Canon PowerShot G6 digital device.

Apoptosis analyses. Cells were seeded at a density of $2x10^5$ cells/well onto chamber slides or 6-well plates (both from Nunc). After overnight attachment, cells were cultured for 48 h in the presence or absence of 10 μg/ml BA. For immunofluorescent staining of cleaved caspase 3, cells were fixed in chamber slides for 20 min in fresh 4% paraformaldehyde at room temperature, permeabilized in 0.1% Triton X-100/0.1% sodium citrate, blocked in 5% BSA and incubated overnight at 4˚C with a polyclonal rabbit anti-human cleaved caspase 3 antibody (Cell Signaling Technology). Cells were then incubated for 30 min with FITC-conjugated sheep anti-rabbit IgG (1:320, Sigma-Aldrich, Hamburg, Germany) at room temperature. Nuclear staining was performed with Vectashield® containing 4,6-diamidino-2-pheylindole (Vector Laboratories Inc., Burlingame, CA, USA). Cleaved caspase 3-positive cells were counted at x200 magnification in at least 10 microscope fields. For annexin V-based apoptosis analysis, cells were trypsinized in 6-well plates, washed with PBS and suspended in 500 μl of calcium-containing binding buffer. Cy5-conjugated annexin V (1:100; BioVision, Mountain View, CA, USA) and 5 μM calcein (Invitrogen, Carlsbad, CA, USA) were added to the cell suspension. Early apoptotic cells (annexin V and calcein-positive) were detected using cell fluorescence assays on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Real-time reverse transcription-PCR (RT-PCR). RNA extraction and real-time RT-PCR was performed as previously described (25). We used the following primer pairs (5'->3' orientation): *BCL2*, CGCCCTGTGGATGACTGAGTAC, CAAACAGAGGCCGCATGCT; *Survivin*, GGACAGAGAA AGAGCCAAGAACAA, CCAGCTGCTCGATGGCAC; *GLI1*, AGCTACATCAACTCCGGCCA, GCTGCGGCGTT CAAGAGA; *IGF2*, CCTCCGACCGTGCTTCC, GGTGGA CTGCTTCCAGGTGT; *PTCH1*, TTGATTGTGGGTGG CACAGT, GCTTGGGAGTCATTAACTGGAAC; *TATA box-binding protein* (TBP), GCCCGAAACGCCGAATAT, CCGTGGTTCGTGGCTCTCT. Amplification of the housekeeping gene *TBP* was performed to standardize the amount of sample RNA. Relative quantification of gene expression was performed using the $\Delta\Delta$ct method as described earlier (26).

Statistical analysis. Data were expressed as means + standard deviation and statistically subjected to Student's unpaired t-test. A level of $P<0.05$ was considered to be significant.

Results

Betulinic acid inhibits PI3K/AKT-mediated growth of HB cells. Several lines of evidence indicate that upregulation of *IGF2* is a key event in the development of HB (9). Since *IGF2* is known to activate the PI3K/AKT pathway (11) we wondered whether an autocrine IGF2 regulatory circuit is also activated in HB. Therefore, we have verified the phosphorylation status of the key protein of PI3K/AKT signaling, the serine/threonine kinase AKT. Using Western blot analysis we found a strong activation of AKT in all three HB cell lines under investigation, namely HUH6, HepT1 and HepT3 (Fig. 1). Interestingly, these cell lines originate from different histological subtypes comprising epithelial and mixed HB. In contrast, the HCC cell line HepG2 exhibited only unphosphorylated AKT. This suggests that *IGF2* overexpression, which is consistently found in HB tumors (9), could lead to a constitutive activation of AKT in these tumors independent of their histology. In contrast, in HCC deregulation other than activated PI3K/AKT signaling might be implicated in tumorigenesis.

Since activation of PI3K/AKT signaling is known to trigger downstream responses such as cell survival and inhibition of apoptosis, which leads to the increased ability of cancer cells to survive (11), we next examined whether abrogation of these molecular mechanisms could be used to inhibit HB growth *in vitro*. BA displays a promising agent

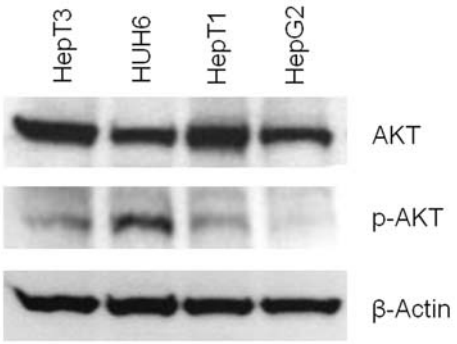

Figure 1. AKT phosphorylation in HB cells. Protein expression levels of AKT and activated phospho-AKT in the HB cell lines HepT3, HUH6 and HepT1, and the HCC cell line HepG2. Protein levels were detected by Western blot analysis using 20 μg of whole cell lysate. The expression of ß-actin protein served as a standard control for equal protein loading.

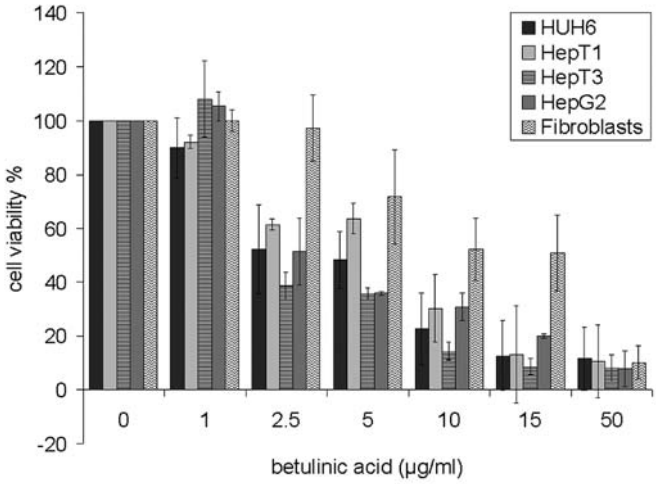

Figure 2. BA treatment causes decreased cell viability of HB cells. The HB cell lines HUH6, HepT1, and HepT3, the HCC cell line HepG2 and normal human fibroblasts were treated for 24 h with increasing BA concentrations. Cell viability was analyzed by MTT assay in three independent experiments and is depicted as the mean percentage ± standard deviation of viable cells.

known to exert a strong cytotoxic effect on a variety of human cancers by inhibiting growth and inducing apoptosis (14). By applying BA concentrations (0-50 μg/ml) comparable to those used in previous studies (13,17) we observed a dose-dependent decrease in cell viability in all tumor cell lines by means of MTT assay (Fig. 2). This effect was selective for tumor cells, since normal fibroblasts were hardly responsive to BA treatment. However, high concentrations of BA (50 μg/ml) had a strong cytotoxic effect and resulted in complete killing of all cell lines. The half-maximal cytotoxic concentration (IC_{50}) of BA at 24 h was 4.70 μg/ml, 6.95 μg/ml, 2.19 μg/ml, 2.89 μg/ml and 23.91 μg/ml for HUH6, HepT1, HepT3, HepG2 and normal fibroblasts, respectively. Taken together our results clearly demonstrate that BA is highly effective against excessive growth of childhood liver tumor cells.

BA selectively induces apoptosis in HB cells. Since it is known that BA exerts its anti-tumorigenic effect by inducing apoptosis (13,17,23), we next verified whether BA can induce apoptotic characteristics in HB cells. First, we screened HB cells after BA treatment for apoptosis-specific morphological changes, such as cell shrinkage, membrane blebbing, nuclear fragmentation and formation of apoptotic bodies. Strikingly, BA treatment resulted in a strong increase of apoptosis in the three tested HB cell lines, but not in the HCC cell line HepG2 (Fig. 3a). We then analyzed membrane integrity by annexin V staining in BA-treated HB cells. Consistent with the morphological changes of HB cells we found significantly elevated external appearance of phosphatidylserine in HB cells, whereas HCC cells showed almost no induction of membrane asymmetry (Fig. 3b). Thereafter, we examined a later stage of apoptosis characterized by proteolytic cleavage of caspase 3 by immunofluorescence. In the presence of BA cleaved caspase 3-positive cells were markedly increased only in HB cell lines (Fig. 3c). Consistently, using Western blot analysis we detected increased levels of the activated form of caspase 3 and its known downstream target poly-(ADP-ribose) polymerase in the HB cell lines, which was most prominent in the 10 μg/ml BA treatment samples (Fig. 3d). Contrarily, the HCC cells lack activated forms of both apoptosis-related proteins. Altogether, these findings suggest that BA treatment effectively induces apoptosis in HB cells.

However, the reason for the inability of the HCC cells to undergo apoptosis upon BA treatment has prompted us to investigate the molecular basis for this. It is known that elevated expression of survival genes could confer resistance to apoptosis of tumor cells (27-29). Therefore, we checked the expression of the known anti-apoptosis genes *BCL2* and *Survivin* by real-time RT-PCR. We found that HepG2 cells exhibit significant higher levels of *BCL2* (7-fold) and *Survivin* (2.5-fold) compared to HB cells (Fig. 3e), thus emphasizing that inhibition of apoptosis by overexpression of survival genes is one possible mechanism for the resistance of these cells against BA.

BA inhibits known target genes of hedgehog signaling. Deregulation of hedgehog signaling plays a fundamental role in an increasing number of malignancies, including solid childhood tumors (30,31). We recently described that overexpression of hedgehog target genes such as *GLI1* and *PTCH1* are frequently found in HB (25). Since HepT3 cells display an activated hedgehog signaling pathway (25), we wondered whether BA has any impact on the signaling cascade in these cells. Using real-time RT-PCR analysis for the known hedgehog target genes *GLI1*, *PTCH1* and *IGF2* (31), we found a significant reduction of mRNA levels for all three tested genes in HepT3 cells after BA treatment (Fig. 4). In contrast, the HCC cell line HepG2, which has already been reported to lack activation of the hedgehog signaling pathway (32), showed no relevant changes in target gene expression. These results suggest that BA is able to inhibit hedgehog signaling at least on the transcriptional level.

Discussion

The current therapy protocol for HB is based on systemic chemotherapy and surgery and strives for removal of the entire tumor, because cure without complete resection is

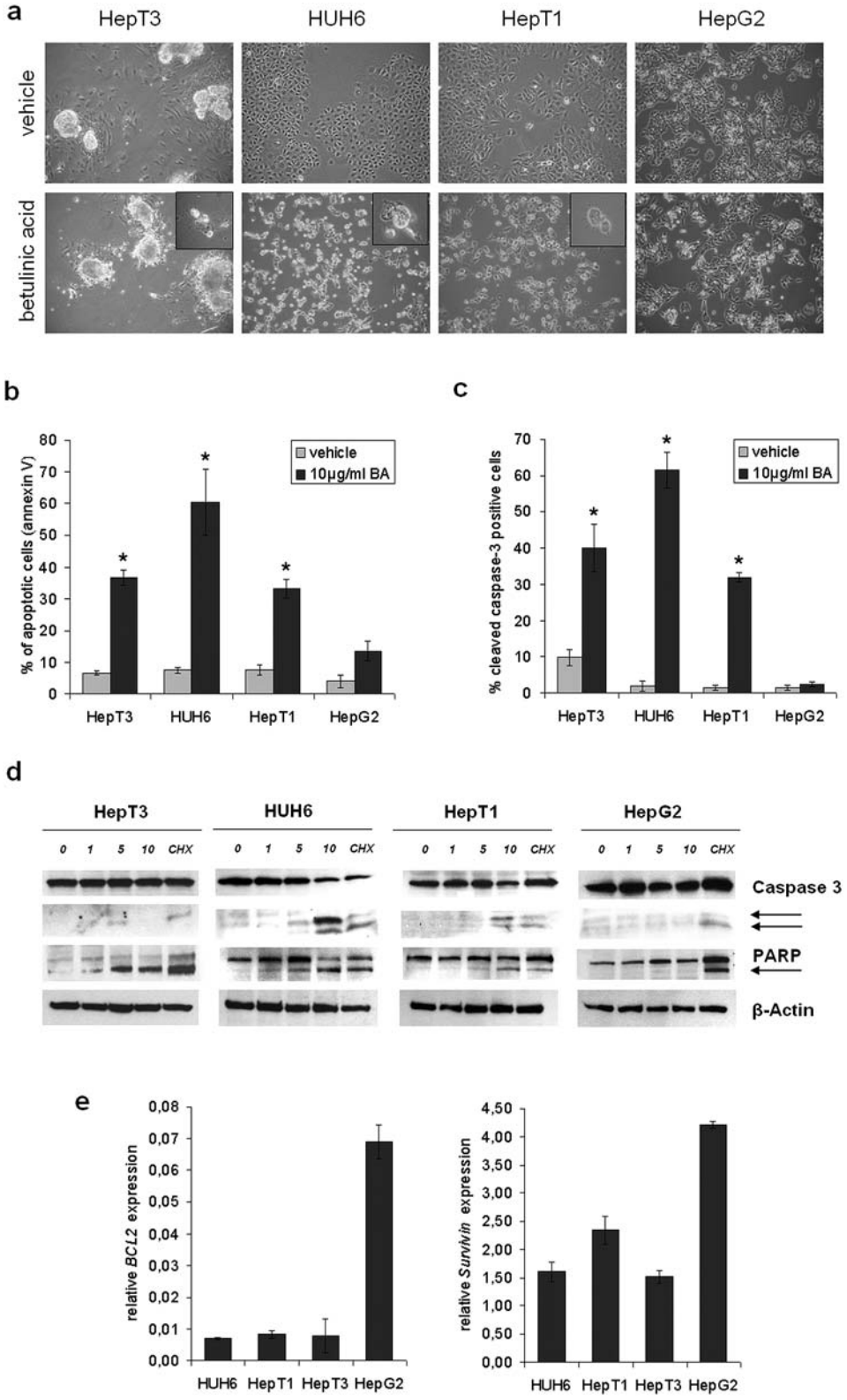

Figure 3. Apoptosis induction in BA-treated HB cell lines. (a) Morphology. HB cells (HepT3, HUH6 and HepT1) and a HCC cell line (HepG2) were treated for 24 h with or without 10 μg/ml BA. Typical morphological changes after apoptosis induction were observed in all three HB cell lines, but not in the HCC cell line. Insets show apoptotic cells at high magnification. (b) Annexin V staining. Cells were treated for 24 h with 10 μg/ml BA or vehicle followed by apoptosis assay using Cy5-conjugated annexin V and calcein. Results are means ± standard deviation of two experiments performed in duplicates and are expressed as percentages of early apoptotic cells (annexin V and calcein-positive). Statistical significant difference versus vehicle; *P<0.05 (unpaired Student's t-test). (c) Activation of caspase 3. Apoptotic cells were counted under a fluorescence microscope after 48 h incubation with 10 μg/ml BA using immunofluorescent detection of cleaved caspase 3 and nuclear counterstaining. Percentages of cleaved caspase 3-positive cells are depicted. Statistical significant difference versus vehicle; *P<0.05 (unpaired Student's t-test). (d) Western Blot analysis. HB cells were treated for 24 h with 10 μg/ml BA as indicated and expression of caspase 3, poly(ADP-ribose) polymerase (PARP), and ß-actin was detected by Western blot analysis. Cleaved caspase 3 and PARP are indicated by arrows. Cycloheximide (100 μg/ml; CHX) was used as a positive control for apoptosis induction. Immunodetection of ß-actin served as a standard control for equal protein loading. (e) Real-time RT-PCR. The mRNA expression of the anti-apoptotic genes *BCL2* and *Survivin* was measured in HB and HCC cells by real-time RT-PCR in relation to the house-keeping gene *TBP* as a calibrator.

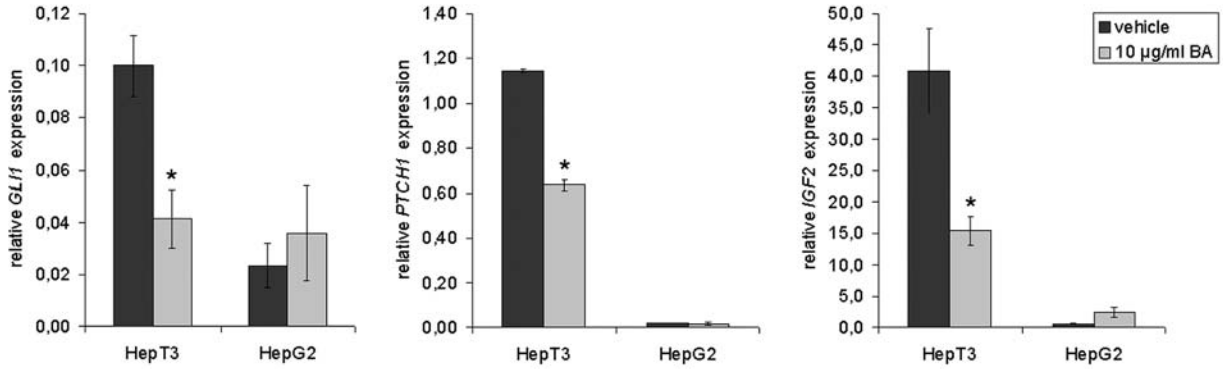

Figure 4. BA interferes with hedgehog signaling. Hedgehog-activated HepT3 and hedgehog-non-activated HepG2 cells were treated for 24 h with 10 μg/ml BA. *GLI1*, *PTCH1* and *IGF2* mRNA expression from untreated (black bars) and BA-treated (grey bars) cells was measured by real-time RT-PCR. *TBP* expression was used as a calibrator. Statistical significant difference versus vehicle; $^{*}P<0.05$ (unpaired Student's t-test).

extremely unusual (33). Preoperative debulking of the tumor using chemotherapy still constitutes a major challenge especially in HB patients with advanced disease (5). Thus, new strategies to target tumor cells are essential to improve patient outcome. In the present study, we provide clear evidence that BA could be used as a powerful anti-tumoral agent to effectively inhibit HB growth *in vitro*. We could furthermore demonstrate that BA strongly induces apoptosis in HB cells. Additionally, BA treatment led to a significant decrease in expression of the hedgehog target genes *GLI1*, *PTCH1* and *IGF2* in the hedgehog-activated HB cell line HepT3. Together, these data indicate that BA might be a hopeful new agent for treating HB, especially those with an activated hedgehog signaling pathway.

Activation of the PI3K/AKT pathway is a common feature of a variety of human cancers, especially those of early childhood (11,34). Phosphorylation of the serine/threonine kinase AKT is known to trigger inactivation of proapoptotic factors, which in turn confers a survival advantage to tumor cells (12). Since our study clearly shows that AKT is activated in all HB cell lines under investigation independent of their histology, it is safe to predict that *IGF2* overexpression, which is frequently found in HB (6,9), leads to an autocrine IGF2 regulatory circuit that constitutively drives HB growth. Based on this assumption it is intriguing that BA treatment is able to effectively inhibit growth and induce apoptosis in HB cells. Our data stand in line with studies on other childhood tumors such as medulloblastoma, neuroblastoma, glioblastoma and Ewing's sarcoma (16,22,23). The finding that the level of proteolytically cleaved caspase 3 dramatically increased in HB cells upon BA treatment suggests that BA induces apoptosis through the intrinsic mitochondrial pathway. This is consistent with previous reports convincingly demonstrating that BA-induced apoptosis is mediated by acting directly on the mitochondria (17,18). Thus, the mode of BA action seems to be the same in a variety of cancers. This is particularly intriguing in view of treating tumors such as HB in which upstream components of apoptosis-inducing signaling pathways are interrupted. In view of a clinical setting one has to realize that all studies conducted so far used a comparable BA concentration of 5-20 μg/ml. Most importantly, these BA levels could be reached in the serum of mice after intraperitoneal administration and were tolerated without toxic side-effects (35). In this context it is worth mentioning that BA treatment in our study had a selective effect on tumor cells leaving normal fibroblasts grossly viable, which corroborates previous studies using proliferating normal lymphocytes and mouse fibroblasts (15,19). Taken together, our successful strategy of blocking IGF2-mediated cancer cell survival by inducing apoptosis might display a rationale for the future treatment of HB. Since it has been reported that BA could sensitize cancer cells for anticancer drugs (36), BA may be used in combination to established treatment regimens in future therapeutic approaches.

However, the inability of the HCC cell line HepG2 to undergo apoptosis upon BA treatment also point to some limitation of this agent. We found at least one explanation for the resistance of these tumor cells, namely the upregulation of the anti-apoptosis genes *BCL2* and *Survivin*. This is in accordance with previous studies showing that *BCL2* as well as *Survivin* could inhibit the intrinsic mitochondrial apoptosis pathway (37,38). To circumvent the insensitivity to chemotherapeutic drugs or induction of apoptosis in pediatric liver cancer cells several attempts have already been made to modulate the intrinsic expression of *BCL2* and *Survivin*, showing the importance of these two anti-apoptotic proteins in inhibiting apoptosis (39-41). Altogether these data suggest that decreasing the expression levels of survival genes combined with conventional chemotherapeutical treatment might be a promising approach to overcome the prevalent resistance of tumors.

An additional intriguing result of the present study is that BA has an impact on the hedgehog signaling pathway. Deregulation of hedgehog signaling plays a fundamental role in an increasing number of malignancies (30,31), and we have recently added HB to the list (25). Here, we report on the transcriptional repression of the known hedgehog target genes *GLI1*, *PTCH1* and *IGF2* in hedgehog-activated HepT3 cells, while HepG2 known to lack activation of the hedgehog signaling pathway (32) showed no reduction in target gene expression. These results are in line with a recent study of Arai *et al* (42), which suggests that BA is able to inhibit hedgehog signaling on the level of the transcription factor GLI1. GLI-dependent luciferase reporter assays were used for screening a natural resource library for hedgehog-

inhibiting compounds. Among others BA had the strongest inhibitory effect on GLI1-mediated transcriptional activity. Selective targeting of the hedgehog pathway is nowadays heavily discussed to add therapeutic benefit to current cancer treatments (reviewed in ref. 43). This hope was fueled by the first promising preclinical studies on digestive tract tumors, pancreatic and small-cell lung cancer as well as medulloblastoma, in which blocking the hedgehog pathway consistently showed a dramatic retardation of tumor growth *in vivo* (reviewed in refs. 30,31). Clinical phase I and II studies using hedgehog inhibitors for advanced and/or metastatic solid tumors are currently underway.

In summary, our results convincingly show that BA treatment causes extensive apoptosis and inhibition of hedgehog signaling in HB. Since BA obviously is effective in a growing number of cancers the use of BA or more potent derivatives might be a promising approach for clinical applications in the treatment of hedgehog-dependent cancers, including pediatric liver tumors.

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