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Synergistic induction of cell death in liver tumor cells by TRAIL and chemotherapeutic drugs via the BH3-only proteins Bim and Bid

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Although death receptors and chemotherapeutic drugs activate distinct apoptosis signaling cascades, crosstalk between the extrinsic and intrinsic apoptosis pathway has been recognized as an important amplification mechanism. Best known in this regard is the amplification of the Fas (CD95) signal in hepatocytes via caspase 8-mediated cleavage of Bid and activation of the mitochondrial apoptosis pathway. Recent evidence, however, indicates that activation of other BH3-only proteins may also be critical for the crosstalk between death receptors and mitochondrial triggers. In this study, we show that TNF-related apoptosis-inducing ligand (TRAIL) and chemotherapeutic drugs synergistically induce apoptosis in various transformed and untransformed liver-derived cell lines, as well as in primary human hepatocytes. Both, preincubation with TRAIL as well as chemotherapeutic drugs could sensitize cells for apoptosis induction by the other respective trigger. TRAIL induced a strong and long lasting activation of Jun kinase, and activation of the BH3-only protein Bim. Consequently, synergistic induction of apoptosis by TRAIL and chemotherapeutic drugs was dependent on Jun kinase activity, and expression of Bim and Bid. These findings confirm a previously defined role of TRAIL and Bim in the regulation of hepatocyte apoptosis, and demonstrate that the TRAIL–Jun kinase–Bim axis is a major and important apoptosis amplification pathway in primary hepatocytes and liver tumor cells.

Specific and selective apoptosis induction in tumor cells is a major goal of cancer therapy.[1,2] Most currently known treatments target in one or the other way the apoptosis machinery of tumor cells. However, apoptosis resistance is one of the hallmarks of cancer, and a prerequisite of transformed cells to develop into a tumor.[3] Consequently, many tumor cells are highly resistant to a variety of antitumor drugs, which limits the treatment option in cancer patients. Thus, novel therapeutics, possibly targeting alternative signaling pathways and thereby bypassing apoptosis resistance, are urgently needed and the focus of intense research. Since its discovery the tumor necrosis factor family member TNF-related apoptosis-inducing ligand (TRAIL/TNFSF2) has received extensive attention because of its relatively selective induction of cell death in tumor cells.[4] TRAIL promotes apoptosis in a variety of tumors of different origin, but has usually no effect on primary cells, at least under physiological conditions. For example, Walczak et al.[5] demonstrated that treatment of mice transplanted with human tumor cells resulted in growth inhibition of transplanted tumors or their eradication. In contrast, no TRAIL-induced toxicity was observed in tissue cells, for example, hepatocytes, suggesting that TRAIL may be a useful and safe future antitumor drug.

Most chemotherapeutic drugs activate the mitochondrial apoptosis pathway, and are thus selectively inhibited by high levels of antiapoptotic Bcl-2 homologs frequently seen in tumor cells. In contrast, in most cells TRAIL activates the caspase cascade directly at the receptor complex and thereby bypasses a potential mitochondria-restricted apoptosis resistance (type I cells). TRAIL may therefore induce death in cells that are resistant to other apoptosis-inducing drugs. Another interesting aspect of TRAIL is its synergy with triggers of the mitochondrial pathway, for example, chemotherapy or irradiation. Although many tumor cells show relative resistance to either TRAIL or a given chemotherapeutic drug, the combined treatment of tumor cells with both triggers often leads to a synergistic and efficient induction of cell death. This synergy between the death receptor and the mitochondrial pathway has been observed in lung cancer cells, colon carcinoma and other types of tumors.[6–8]

Although the synergistic induction of apoptosis by combined treatment with TRAIL and chemotherapeutic drugs is well established, their underlying mechanisms are incompletely understood. It has been suggested that DNA damage will lead to an increase in TRAIL receptor expression via the activation of the tumor suppressor and transcription factor

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p53, and thereby to increased sensitivity to TRAIL-induced apoptosis.[9] Indeed, TRAIL receptor 2 is a p53-responsive target gene.[10] As the synergy between TRAIL and chemotherapy is also found in p53 mutant cells,[6] the p53-mediated induction of TRAIL receptor 2 is likely an incomplete explanation. Other studies have suggested that the TRAIL- and chemotherapy-induced c-Jun kinase (JNK) pathway may have an important role in the synergistic induction of cell death.[11]

Although primary cells are mostly resistant to TRAIL-induced apoptosis, there is increasing evidence that TRAIL can modulate and enhance apoptosis induced by other triggers even in primary cells. Of particular interest in this regard is the role of TRAIL in hepatocyte apoptosis. Although *in vivo* administration of the TNF homolog Fas ligand causes rapid death because of the induction of excessive liver damage, therapeutic doses of TRAIL seem to be tolerated well.[5] We recently described that TRAIL fails to trigger apoptosis in primary hepatocytes but enhances their sensitivity to the Fas pathway.[12] Synergistic induction of hepatocyte apoptosis *in vitro* and liver damage *in vivo* was found to be dependent on TRAIL-induced activation of JNK and the pro-apoptotic Bcl-2 homolog Bim. Interestingly, a similar pathway has been described for TNFα-mediated liver damage,[13] suggesting that the JNK–Bim axis is an important response modifier pathway.

In this study, we investigated the role of the JNK–Bim axis in the synergistic induction of apoptosis by TRAIL and chemotherapeutic drugs in human liver tumor cells and hepatocytes. Our data confirm the potentiation of the mitochondrial pathway by TRAIL in hepatoma cell lines, hepatocellular carcinoma cell lines, immortalized hepatocytes as well as primary human hepatocytes. TRAIL but not chemotherapy induced a strong activation of JNK and subsequent phosphorylation of Bim. Synergistic induction of apoptosis by TRAIL and doxorubicin was associated with increased binding of Bim to Mcl-1, activation of Bax, release of Cytochrome *c* and SMAC from the mitochondria, and increased activation of caspases. Inhibition of JNK, knock-down of Bim and Bid by RNA interference, or overexpression of Mcl-1 and Bcl-x_L efficiently inhibited cell death induced by the combined treatment of cells with TRAIL and chemotherapy. These findings demonstrate that TRAIL-JNK-Bim axis is a major and important apoptosis amplification pathway in primary hepatocytes and liver tumor cells.

Results

Synergistic induction of apoptosis by TRAIL and doxorubicin in liver tumor cells. Synergistic induction of cell death by TRAIL and chemotherapeutics has been described in different tumors cell lines.[6–8,14] Similarly, we have previously reported that TRAIL can enhance the Fas-induced apoptosis pathway in hepatocytes via a JNK–Bim-dependent pathway.[12] To investigate whether the TRAIL-initiated JNK–Bim pathway has also major role in the induction of cell death by TRAIL and chemotherapy, we assessed cell death induced by TRAIL and doxorubicin in different liver tumor cell lines as well as immortalized human

hepatocytes (IHHs). Figure 1a illustrates that doxorubicin was found to be an inefficient inducer of cell death in HepG2 and Huh7 cells, and only a weak inducer of apoptosis in Hep3B and IHH cells. Similarly, only weak induction of cell death was seen in these cell lines with TRAIL concentrations up to 50 ng/ml. In marked contrast, when cell lines were preincubated with 10 ng/ml TRAIL for 30 min before the treatment with increasing concentrations of doxorubicin a profound sensitization and strongly increased cell death induction was seen in all cell lines. Interestingly, an identical sensitization was seen when cells were preincubated for 30 min with 1 μg/ml doxorubicin and then stimulated with TRAIL. This synergistic induction of cell death was confirmed by detection of caspase activity (DEVD cleavage; Figure 1b) and annexin V staining (Figure 1c). These data confirm that TRAIL and doxorubicin synergistically induce apoptosis in different liver-derived cell lines.

Synergistic induction of cell death is not dependent on preconditioning. We next investigated whether preincubation with either doxorubicin or TRAIL was required for the synergistic enhancement of cell death induction by the respective other agonist. HepG2 cells were thus pretreated with low doses of TRAIL or doxorubicin for 60 min, before stimulation with increasing concentrations of doxorubin or TRAIL. Alternatively, both triggers were added simultaneously. Interestingly, the synergistic induction of cell death by TRAIL and doxorubicin was as efficient when pretreated for 60 min with one agonist and then treated with the other, or when triggers were added at the same time. Similarly, no difference was seen whether cells were preconditioned by TRAIL or doxorubicin (Figure 2).

TRAIL synergizes with different mitochondrial triggers. To further explore whether this synergistic induction of cell death was restricted to the combination TRAIL–doxorubicin, or further extends to other apoptosis triggers, HepG2 cells were preconditioned with medium control or low concentrations of TRAIL, and then exposed to staurosporin, irinotecan (CPT-11), 5 fluorouracil or cisplatin. Remarkably, although most of these agonists alone were relatively inefficient triggers of apoptosis in HepG2 cells, pretreatment with TRAIL greatly sensitized cells to apoptosis induction by these mitochondrial triggers (Figure 3). Similar findings were made for Hep3B cells (data not shown).

Role of JNK in synergistic induction of cell death. We previously described that in murine hepatocytes, TRAIL synergistically enhanced the Fas apoptosis pathway via a JNK–Bim-dependent process.[12] The role of JNK activation was thus analyzed in TRAIL-mediated enhancement of chemotherapy-induced cell death in liver tumor cells. HepG2 cells were treated with doxorubine, TRAIL or the combination thereof for various time points, and the activation of JNK was monitored by the detection of phospho-JNK (Figure 4a). Notably, whereas both doxorubicin and TRAIL induced JNK activation within 30 min, TRAIL seemed to be a more potent inducer of JNK activation. Combined treatment of cells with TRAIL and

doxorubicin did not result in a substantial increase in JNK activation.

HepG2 cells were then pretreated with increasing concentrations of JNK inhibitor II, and apoptosis sensitivity to the combination of TRAIL and doxorubicin was analyzed.
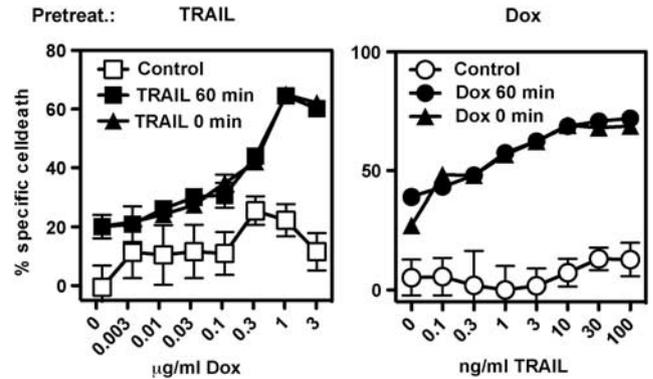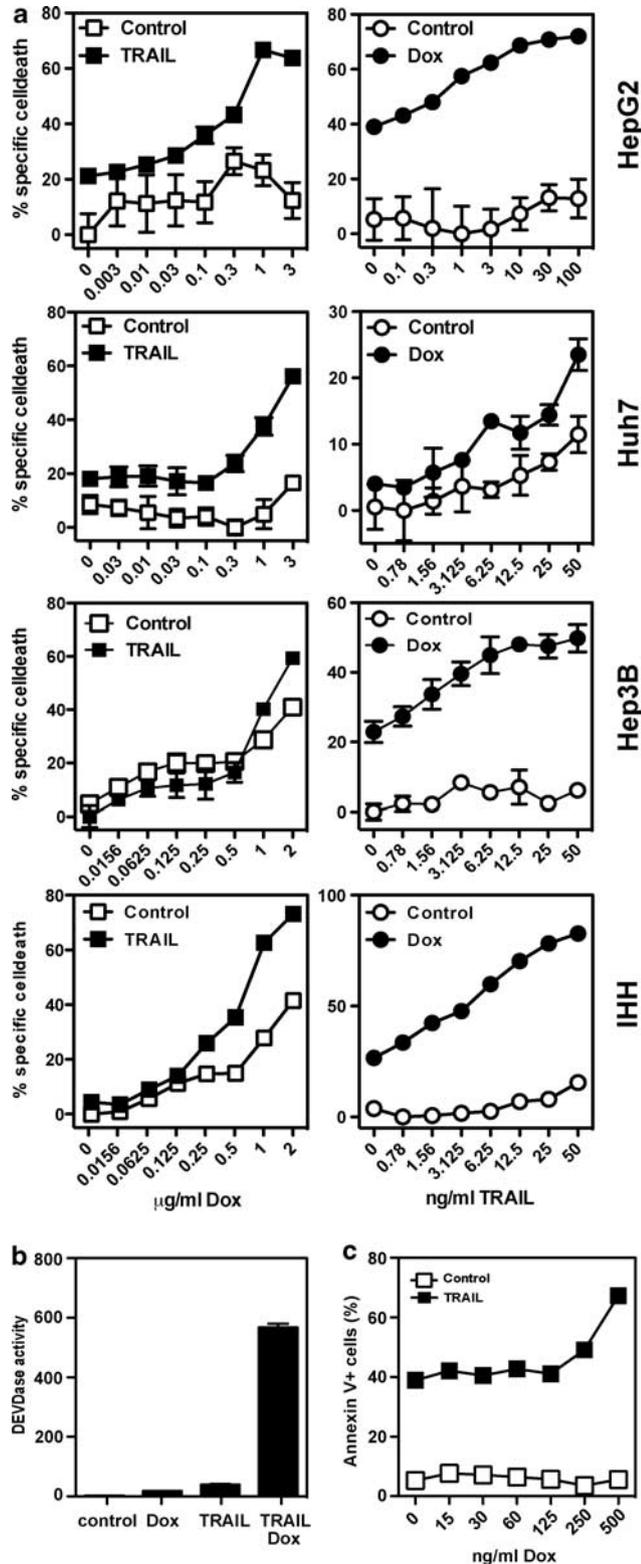

Figure 2 Effects of pretreatment on synergistic induction of cell death. HepG2 cells were pretreated with medium control, TRAIL (left panel, 10 ng/ml) or doxorubicin (Dox, right panel, 1 μg/ml), either 60 min before adding increasing doses of doxorubicin or TRAIL, or directly together with the apoptosis trigger (0 min). Cell death was measured by MTT assay. Mean values ± S.D. of quadruplicates are shown. n = 2
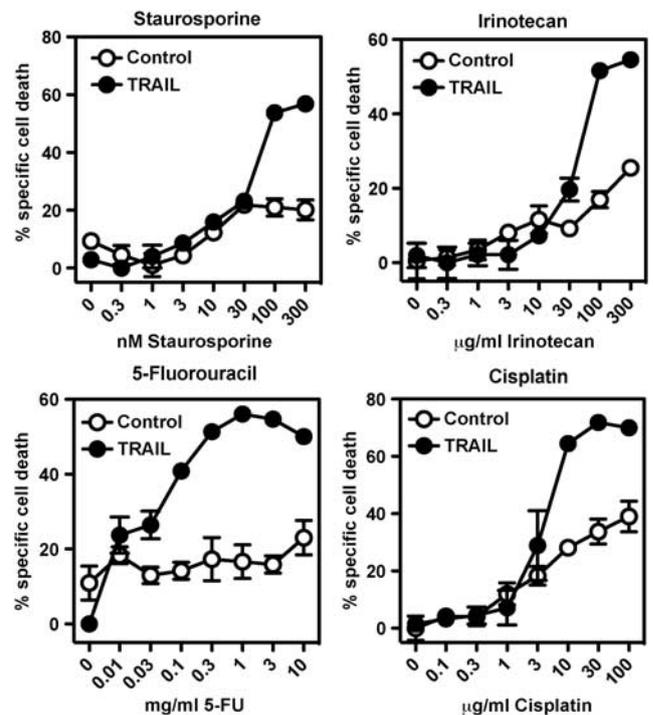

Figure 3 TRAIL synergizes with different chemotherapeutic drugs. HepG2 cells were either pretreated with medium control or 10 ng/ml TRAIL, and then exposed to increasing concentrations of staurosporin, irinotecan, 5-fluorouracil (5-FU) or cisplatin. Cell death was measured by MTT assay. Mean values ± S.D. of quadruplicates are shown. n = 2

Figure 1 Synergistic induction of cell death by doxorubicin and TRAIL. (**a**) HepG2, Huh7, Hep3B and IHH cells were pretreated by either medium control, 10 ng/ml TRAIL (left panels) or 1 μg/ml doxorubicin (Dox, right panels), before the exposure to increasing concentration of doxorubicin (left panels) or TRAIL (right panels). Cell death was measured by MTT assay. Mean values ± S.D. of quadruplicates of a typical experiment (n = 3) are shown. (**b**) HepG2 cells were treated with 1 μg/ml doxorubicin, 10 ng/ml TRAIL or the combination thereof for 6 h, and DEVDase activity in cell lysates was measured. Mean values ± S.D. of quadruplicates were measured. n = 3. (**c**) HepG2 cells were pretreated with medium control or 10 ng/ml TRAIL, and then exposed to increasing doses of doxorubicin. Apoptosis was measured by Annexin V binding. n = 2
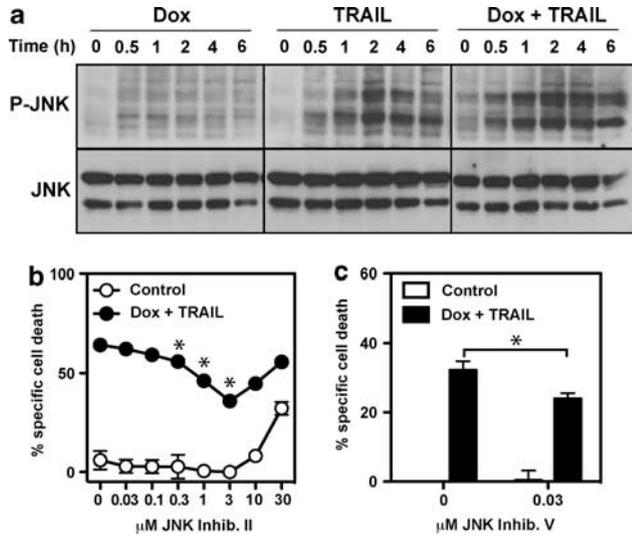
Figure 4 Role of JNK in TRAIL plus doxorubicin-induced cell death. (**a**) HepG2 cells were treated for indicated time with doxorubicin (Dox, 1 μg/ml), TRAIL (10 ng/ml) or the combination of both. Phosphorylated JNK (P-JNK) and total JNK as loading control were detected by western blot. (**b**) Cells were pretreated for 30 min with increasing concentrations of JNK inhibitor (inhib.) II and then exposed to medium control or 1 μg/ml doxorubicin plus 30 ng/ml TRAIL. (**c**) Cells were pretreated with 0.03 uM JNK inhibitor V, and then exposed to medium control or doxorubicin plus TRAIL. Cell death was measured by MTT assay, mean values $\pm$ S.D. of quadruplicates are shown. $n = 3$. *$P < 0.01$ (control treated versus JNK inhibitor treated)

Although the JNK inhibitor II was found to be toxic at concentrations 10 μM or higher, it dose dependently inhibited the synergistic induction of cell death by TRAIL and doxorubicin at lower doses (Figure 4b). A similar inhibition of cell death induction by TRAIL plus doxorubicin was seen when cells were treated with the JNK V inhibitor (Figure 4c). These data suggested that JNK is involved in the sensitization of cells for apoptosis induction.

TRAIL promotes Bim phosphorylation. We[12] and others[13] have previously identified Bim as an important target of JNK in murine hepatocytes. The phosphorylation of Bim in response to doxorubicin, TRAIL, or the combination of both was thus analyzed in HepG2 cells. Interestingly, Bim_{EL} was hardly detectable in NP-40 lysates of untreated control cells, but became rapidly mobilized after treatment with doxorubicin or TRAIL (Figure 5a). Likely, this represents a release of Bim_{EL} from intracellular (NP40-resistant) stores, such as the cytoskeleton,[15] as Bim_{EL} was detected at high levels in SDS lysates of control cells (Figure 5b). Although in doxorubicin-treated cells Bim_{EL} remained predominantly in its unphosphorylated form, treatment of cells with TRAIL resulted in a time-dependent increase of higher molecular weight variants, indicative of Bim_{EL} phosphorylation.[12,13,16] Although Bim_{EL} levels remained stable in doxorubicin- or TRAIL-stimulated cells, combined treatment with doxorubicin and TRAIL resulted in the disappearance of Bim_{EL}, indicating consumption of Bim in dying cells (Figure 5b).

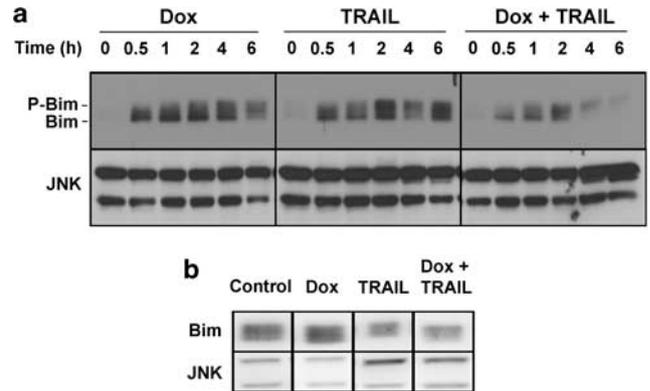**JNK induces activation of Bim.** Previous studies have indicated that the JNK-mediated phosphorylation represents

Figure 5 TRAIL promotes Bim mobilization and phosphorylation. (**a**) HepG 2 cells were treated for indicated time with 1 μg/ml doxorubicin (Dox), 10 ng/ml TRAIL, or the combination thereof. Bim levels in NP40 lysates were analyzed by western blot. Detection of JNK was used for loading control. Positions of Bim and phosphorylated Bim (P-Bim) are shown. (**b**) Cells were treated as indicated above for 4 h, lyzed in SDS buffer and total Bim and JNK levels were analyzed by western blot

an activation step for Bim_{EL} and downstream apoptosis pathway.[12,13,16–18] As Bim_{EL} strongly binds and neutralizes the antiapoptotic Bcl-2 homolog Mcl-1,[19] we analyzed the apoptosis trigger-induced binding of Bim_{EL} to Mcl-1 in the presence of a JNK inhibitor. When Mcl-1 was immunoprecipitated and bound Bim_{EL} was detected by western blotting, a clear increase in Bim_{EL} binding to Mcl-1 was noted in response to TRAIL and doxorubicin treatment. In contrast, no increase in Bim binding to Mcl-1 was found in JNK inhibitor-pretreated cells (Figure 6a). Total Bim_{EL} and Mcl-1 levels were similar under these conditions, apart from reduced levels of Mcl-1 in TRAIL and doxorubicin-treated cells in the absence of JNK inhibitor, likely representing Mcl-1 degradation in dying cells.[20]

TRAIL and chemotherapy synergistically activate the mitochondrial pathway. The synergistic activation of Bim by TRAIL and doxorubicin suggested a strong activation of the mitochondrial apoptosis pathway. Activation of Bax, a pro-apoptotic Bcl-2 homolog critically involved in the permeabilization of the outer mitochondrial membrane and the release of Cytochrome c, was therefore analyzed by immunoprecipitation of active Bax using an anti-N-terminus antibody under native conditions. Although no active Bax could be pulled down in control or doxorubicin-treated cells, and only low levels in TRAIL-treated cells, combined treatment with TRAIL and doxorubicin resulted in a strong increase of Bax activation (Figure 6b). This increased Bax activation was paralleled with an increased mitochondrial outer membrane permeabilization and release of Cytochrome c and SMAC (Figure 6c). In agreement with an induction of the mitochondrial apoptosis pathway, doxorubicin plus TRAIL-induced caspase activation was efficiently blocked by the overexpression of Mcl-1 and Bcl-x_L (Figure 6d).

Critical role for Bim and Bid in the synergistic induction of cell death by TRAIL and doxorubicin. Hepatocytes and hepatocyte-derived cells are known to require the caspase
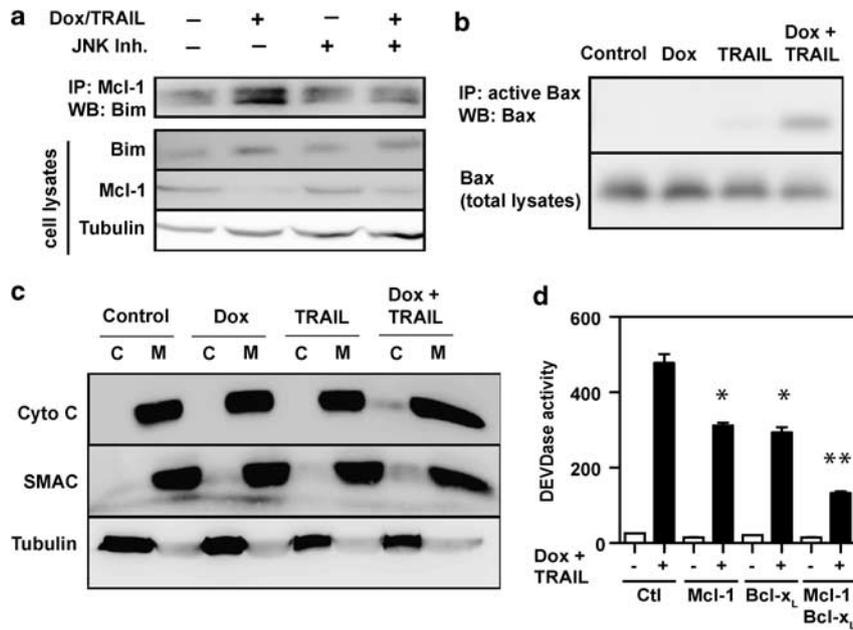
Figure 6 TRAIL plus chemotherapy activates the mitochondrial apoptosis pathway. (**a**) HepG2 cells were pretreated with or without JNK inhibitor (Inh) and the stimulated with medium control (Ctl) or doxorubicin (Dox; 1 μg/ml) plus TRAIL (10 ng/ml). Cells were lysed, Mcl-1 was immunoprecipitated and Bim bound to Mcl-1 was detected by western blot. Bim, Mcl-1 and tubulin levels in total cell lysates were analyzed for comparison. (**b**) Cells were treated with doxorubicin, TRAIL or both, and active Bax was immunoprecipitated and detected by western blot. Bax in total lysates was analyzed for comparison. (**c**) Cells were treated with doxorubicin, TRAIL or both, and cytoplasmic (C) and mitochondrial fractions (M) were isolated. Cytochrome c (Cyto c), SMAC and for comparison tubulin in the different fractions were analyzed by western blot. (**d**) Cells were transfected with control plasmid, Mcl-1, Bcl-xL or both, and then exposed to medium control or doxorubicin plus TRAIL. After 6 h DEVDase activity in cell lysates was analyzed. Mean values $\pm$ S.D. of quadruplicates are shown. $n = 3$. *$P < 0.005$, **$P < 0.001$, compared with control transfection

8-mediated cleavage of the BH3-only protein Bid in order to amplify death receptor signals via the mitochondrial pathway (type II cells).[21,22] We thus investigated whether the synergistic induction of cell death by TRAIL and doxorubicin would also affect the caspase-mediated cleavage and activation of Bid. Although no Bid processing was detectable upon stimulation with TRAIL or doxorubicin alone, the combination thereof caused a time-dependent appearance of truncated Bid, suggesting activation of Bid (Figure 7a).

To assess the relative contribution of the BH3-only proteins Bid and Bim in TRAIL plus doxorubicin-induced cell death, expression of these pro-apoptotic Bcl-2 homologs was reduced by RNA interference (Figure 7b), and caspase activation in response to TRAIL, doxorubicin or both was assessed (Figure 7c). Although downregulation of Bim or Bid alone already resulted in reduced TRAIL plus doxorubicin-induced DEVDase activity, combined knockdown of Bid and Bim was most efficient in preventing caspase activation, indicating that both BH3-only proteins are involved in mediating synergistic induction of cell death by TRAIL and doxorubicin.

TRAIL and doxorubicin sensitize primary hepatocytes. TRAIL has gained considerable interest for the use in cancer therapy because of its selective apoptosis-inducing activity in tumor but not primary tissue cells.[4,5] However, we have recently demonstrated that TRAIL can sensitize primary hepatocytes to Fas-induced apoptosis in a JNK–Bim-dependent manner.[12] As JNK and Bim seem to be

involved also in TRAIL-induced sensitization to chemotherapeutics in hepatocellular carcinomas, we wondered whether TRAIL could also sensitize primary human hepatocytes to doxorubicin-induced apoptosis. As reported previously,[4,5] TRAIL alone did not induce cell death in primary hepatocytes even at very high concentrations (up to 1000 ng/ml). Similarly, even 3 μg/ml doxorubicin failed to promote apoptosis. In marked contrast, even low concentrations of TRAIL strongly sensitized human hepatocytes to doxorubicin-induced cell death, and vice versa (Figure 8). These findings indicate that the synergistic induction of cell death by TRAIL and chemotherapeutics is not restricted to immortalized and tumor cells of the liver, but also extends to primary hepatocytes.

Discussion

Induction of apoptosis is a major goal of various antitumor therapies,[23] however, a hallmark of cancer is also the increased resistance of tumor cells toward apoptosis induction.[3] Consequently, the combined treatment of tumor cells with an apoptosis sensitizer and an apoptosis inducer would appear a perfect strategy to efficiently promote tumor cell death and thus tumor regression. Particularly in this regard TRAIL has received enormous attention. Although many tumor cells are resistant to TRAIL or chemotherapy, various publications in different types of tumors revealed that the combined treatment of tumor cells with TRAIL and chemotherapeutic drugs substantially sensitizes them for apoptosis induction.[6–8,14] As different underlying mechanisms for this
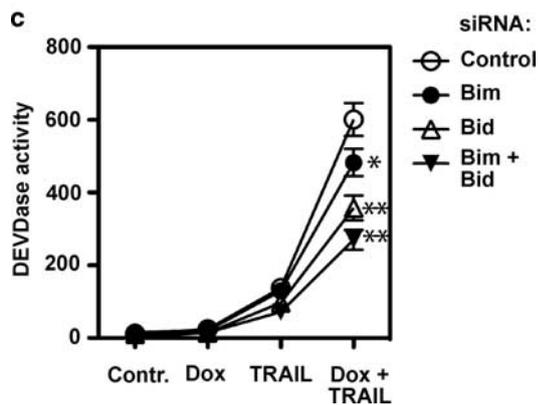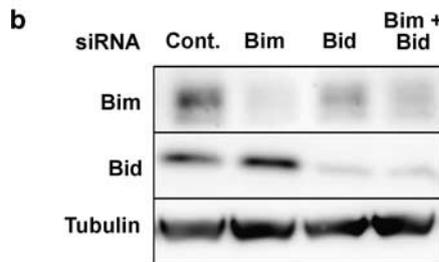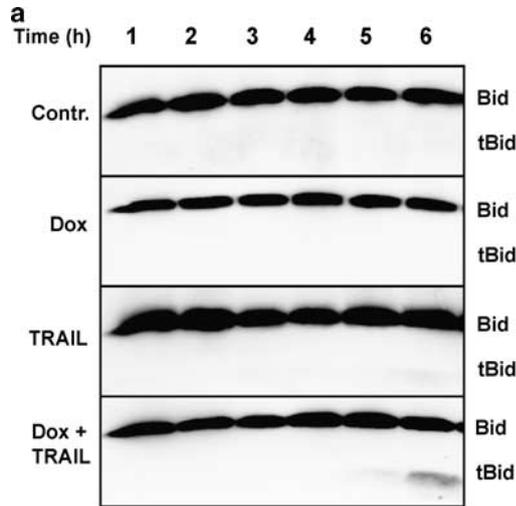
Figure 8 TRAIL plus doxorubicin (Dox) induces cell death in primary human hepatocytes. Primary human hepatocytes were pretreated with 10 ng/ml TRAIL and then exposed to increasing doses of doxorubicin (left panel), or pretreated with 1 μg/ml doxorubicin and then stimulated with increasing doses of TRAIL. Cell death was analyzed after 16 h by MTT assay. Mean values of quadruplicates ± S.D. are shown. $n = 2$

Figure 7 Role of Bid and Bim in TRAIL plus doxorubicin (Dox)-induced cell death. (**a**) HepG2 cells were treated with doxorubicin, TRAIL or both for indicated time intervals and Bid cleavage was analyzed by western blot. (**b**) HepG2 cells were transfected with control (Contr.) siRNA, Bim or Bid specific siRNA or the combination of both. Downregulation of Bim and Bid was analyzed by western blot. Tubulin was used as loading control. (**c**) Cells were treated with siRNA as indicated above and apoptosis sensitivity to doxorubicin (1 μg/ml), TRAIL (10 ng/ml) or both was analyzed by DEVDase clevage assay. Mean values ± S.D. of quadruplicates are shown. $n = 3$. *$P < 0.005$, **$P < 0.0005$ compared with control siRNA. tBid, truncated Bid

are greatly sensitized to apoptosis induction by combined treatment with TRAIL and chemotherapeutic drugs. In line with the above discussed distinction, we have observed that pretreatment with either TRAIL or doxorubicin sensitized tumor cells for apoptosis induction by the respective other trigger. Cell death induction was even comparable when both triggers were added simultaneously, indicating that the sensitizing event occurs rapidly enough to enhance the apoptosis-inducing step. Although this sensitization worked reproducibly in a number of different hepatocellular tumor cell lines, we also found that primary human hepatocytes were equally sensitized to apoptosis induction by the combined treatment with TRAIL and doxorubicin. This was particularly remarkable as none of the triggers alone promoted cell death alone, in line with the reported insensitivity of hepatocytes to TRAIL and chemotherapy.[5,24] This finding points out a potential adverse liver-damaging side effect of combinational tumor therapy with chemotherapeutic drugs and TRAIL. In support of this observation is a recent report demonstrating that the antineoplastic agent 5-azacytidine sensitizes primary human and murine hepatocytes for death receptor-induced apoptosis.[25] Similarly, we have previously demonstrated that Fas-induced hepatocyte apoptosis and liver damage is strongly enhanced by TRAIL.[12] Furthermore, Koschny *et al.*[26] described that primary human and murine hepatocytes are sensitized to TRAIL-induced apoptosis by the proteosome inhibitor bortezomib, though higher concentrations of bortezomib were needed than in hepatocellular carcinoma cells. Regarding the combination doxorubicin and TRAIL we observed a comparable sensitization pattern in hepatocellular tumor cells and primary hepatocytes (Figures 1 and 8). Clearly, all of the findings support the idea that TRAIL plus an additional trigger is required to promote efficient apoptosis in primary hepatocytes. This stands in contrast with the finding of Jo *et al.*,[27] who described that TRAIL alone was sufficient to promote apoptosis in primary human hepatocytes. Differential preconditioning of hepatocyte preparations in the respective donor or by isolation techniques, and associated stress signals, could at least in part explain the observed sensitivity to TRAIL alone.

sensitization have been proposed, it is often not clear which one is the sensitizer and which one is the apoptosis inducer. Likely this distinction is even difficult to be made, or not relevant, as the sensitization to efficient apoptosis induction depends on both, the sensitizer and the apoptosis inducer.

In this study, we have shown that different hepatocellular tumor cells, as well as immortalized and primary hepatocytes,
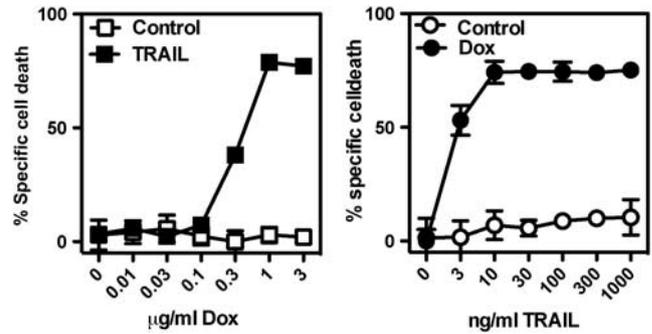
Although it is well established that chemotherapeutic drugs and death ligands strongly synergize in various cell types, the respective mechanisms of sensitization or synergy are not always clear, or may differ from cell type to cell type. Possible mechanisms of chemotherapy-induced enhancement of death receptor-induced apoptosis include p53-induced death receptor expression (e.g., Fas and TRAIL-R2)[28,29] or enhanced death receptor signaling complex assembly and thus increased downstream caspase activation.[30,31] More recent evidence suggests that TRAIL and chemotherapeutic drugs target a common signaling pathway, which involves a JNK-mediated activation or induction of Bim. The BH3-only molecule Bim has an essential role in various forms of apoptosis in different cell types. For example, Bim has been implicated in glucocorticoid receptor-, chemotherapy-, irradia- tion- and growth factor withdrawal-induced apoptosis in tumor cells and primary cells, such as thymocytes.[32] Treatment of cells by apoptosis triggers often leads to an induction of Bim expression, either in an AP-1 or Foxo3a-dependent manner.[33–35] This induction of Bim is particularly evident in glucocorticoid- and T cell receptor-induced apoptosis in thymocytes,[36,37] and Bim-deficient thymocytes show increased resistance to T cell receptor and glucocorticoid- induced apoptosis. However, as many cells constitutively express relatively high levels of Bim in the apparent absence of apoptosis, it is very likely that post-translational modifica- tions significantly contribute to the regulation of Bim-mediated cell death. Thus, it was shown that in unstressed cells Bim is sequestered by the dynein motor complex and released upon exposure of cells to apoptosis triggers.[15,18] In line with this finding, we observed that Bim was hardly detectable in cells lysed with NP40, but became rapidly mobilized (within 30 min) after treatment with TRAIL or doxorubicin (Figure 5). As total Bim levels remained unchanged (in SDS lysates), it is feasible to believe that apoptosis triggers promote a mobilization and redistribution of Bim within the cell, likely promoting transloca- tion of Bim to the mitochondrial membrane, in which it engages other Bcl2 homologs and promotes apoptosis.[15,18]

Another important level of regulation is the phosphorylation of Bim by either ERK or JNK. Whereas phosphorylation of Bim_{EL} by ERK promotes its degradation via the proteosome and thereby enhances cell survival, phosphorylation of Bim_{EL} by JNK seems to enhance its apoptotic activity.[16,17,38] Analysis of apoptosis sensitivity of cells from mutant mice with targeted mutation of the ERK or JNK phosphorylation site in Bim_{EL} supported this model. Fibroblasts with mutated ERK phosphorylation sites showed increased Bim_{EL} stability and increased growth factor withdrawal-induced apoptosis, whereas thymocyte with a mutated JNK phosphorylation site in Bim_{EL} were found to be less sensitive to anti-CD3 and dexamethasone.[38] In line with this notion, we recently observed that Fas-induced hepatocyte apoptosis and liver destruction is substantially regulated via a TRAIL-initiated JNK-mediated activation of Bim.[12] Absence or inhibition of either TRAIL, JNK or Bim resulted in significantly reduced hepatocyte apoptosis. Along these lines, Kaufmann *et al.*[13] found that TNFα-mediated liver destruction was similarly enhanced via the JNK-Bim axis.

In this study, we provide further evidence for the importance of the death receptor–JNK–Bim axis in regulating mitochondrial apoptosis in transformed and primary liver cells. Although we found that both doxorubicin and TRAIL alone were able to promote Bim mobilization from intracellular stores, only combined treatment of cells with TRAIL and chemotherapeutic drugs efficiently triggered cell death. TRAIL treatment of cells resulted in JNK activation and phosphoryla- tion of Bim_{EL}, and in agreement with their respective role in promoting cells death, apoptosis was blocked by JNK inhibitors or knocking down Bim. As TRAIL alone failed to promote apoptosis, this Bim activation step is likely not sufficient, and efficient induction of cell death may require the simultaneous activation of different BH3-only molecules. In agreement with this idea we have seen that Bid was also involved in TRAIL plus doxrubicine induced apoptosis, and combined knockdown of Bim and Bid further reduced apoptosis induction. Other studies in cells from double BH3- only mutant mice support the idea that combined activation of different BH3-only molecules and simultaneous engagement of different downstream effectors may be required for efficient cell death induction.[32,39,40]

In summary, our studies demonstrate a critical role of JNK- mediated Bim activation in the TRAIL-mediated enhancement of chemotherapy-induced apoptosis in liver-derived cells lines, further supporting the importance of the TRAIL-JNK- Bim axis in regulating apoptosis. As the synergistic induction of cell death by TRAIL and doxorubicin was also observed in untransformed primary human hepatocytes, our results also point out potentially deleterious effects of TRAIL combination therapy in promoting undesired liver destruction. Defined manipulation or regulation of these processes may thus help to either enhance tumor therapy or prevent tumor therapy- induced tissue toxicity.

Materials and Methods

Cells and cell lines. The human hepatoma cell line HepG2 (ATCC HB-8065), the human hepatocellular carcinoma cell lines Huh7[41] and Hep3B (ATCC HB-8064), and the IHH cell line IHH[42] were kindly provided by J-F Dufour (Institute of Clinical Pharmacology, Inselspital, University of Bern, Switzerland). Cell were cultured in IMDM containing 5% FCS, 1% L-glutamine and 0.1% gentamycin. The isolation and culture of primary human hepatocytes from human liver specimens has been described previously.[43] Human hepatocytes were cultured in Williams E medium containing 10% FCS, 1% L-glutamine, 0.1% gentamicin, 0.01% insulin/aprotinin and 0.05% transferrin. All media components were from Sigma-Aldrich (Buchs, Switzerland).

Cell death assays. HepG2, Hep3B, Huh7 and IHH were grown in logarithmic phase, harvested and plated in 96-well flat bottom plates. Primary human hepatocytes were directly seeded into collagen-coated 96-well flat bottom plates. After overnight adherence, medium was changed and cells were treated with increasing concentrations of recombinant human TRAIL (untagged form, R&D Systems, Abington, UK) and doxorubicin (Alexis, Lausen, Switzerland), stauro- sporin (Sigma-Aldrich), irinotecan (camptothecin, Alexis), 5-fluorouracil (Valeant, Aliso Viejo, CA, USA), or cisplatin (Alexis) for 16 h. Cell viability was the assessed by MTT assay (Sigma-Aldrich) and normalized to untreated controls, as described previously.[12] Alternatively, cell death was also monitored by Annexin V staining.[44] In some experiments cells were pretreated with Jun kinase inhibitor II or V (Calbiochem, Merck, Darmstadt, Germany) for 30 min, before the induction of cell death.

Caspase activity assay (DEVDase activity). Cells were treated with TRAIL and/or drugs as indicated, and harvested after 6 h. Cells were then washed and lysed for 10 min on ice with 200 ul PBS containing 1% Triton X-100. After centrifugation for 5 min at $16\,000 \times g$ at 4°C, supernatant was harvested and 50 μl were mixed with 150 μl Hepes buffer (100 mM Hepes pH 7.5, 20% glycerol, 0.5 mM

EDTA, 5 mM DTT) containing 100 μM Ac-DEVD-AFC (Alexis). Reactions were incubated for 1 h at 37°C, and enzymatic activity was measured on a spectrofluorometer (400 nm excitation, 505 nm emission). Background fluorescence was measured by incubating caspase substrate with lysis buffer.

Western blotting and immunoprecipitation. After cell death induction for different time intervals and using different trigger combinations, cells were harvested, washed and lysed in cell lysis buffer (1% NP40, 50 mM Tris pH 8.0, 150 mM NaCl, 0.5% deoxycholic acid. In some instances, cells were directly lysed in SDS-PAGE loading buffer containing 1% SDS (total lysates). After removal of insoluble matter and nuclei, equal amounts of protein were separated on a SDS-PAGE and transferred to a nitrocellulose membrane. Specific proteins were then detected using antibodies for JNK, phospho-JNK (both from Cell Signaling Technology, Bioconcept, Allschwil, Switzerland), Bim (Sigma-Aldrich), Bid (BD Biosciences, Basel, Switzerland), Mcl-1 (BD Biosciences) and Bax (Upstate, Milipore, Zug, Switzerland). Equal loading was confirmed by detection of JNK or tubulin.

In some experiments, Mcl-1 was immunoprecipitated using anti-Mcl-1 antibody and Bim bound to Mcl-1 was detected by western blotting. Bax activation was detected by immunoprecipitation of active Bax using an anti-Bax NT antibody (Upstate) and detection by western blotting.

Cytochrome *c* and SMAC release assay. Cytochrome *c* and SMAC release from mitochondria into the cytoplasm was analyzed by western blot as described previously.[45]

Downregulation of Bim and Bid by small interfering RNA (siRNA). Cells were transfected with 10 nM control, Bim- or Bid-specific siRNA (Dharmacon, Lafayette, CO, USA) and 20 ul Hyperfect reagents (Qiagen, Hombrechtikon, Switzerland) in 6 cm culture dishes over night. After washing cells were harvested and redistributed into 96-well plates. After an additional 24 h culture, cells were exposed for 6 h to apoptosis-inducing triggers. Cells were then harvested, lyzed and analyzed for DEVDase activity, and Bim and Bid expression by western blot.

Transfection and overexpression. HepG2 cells were transfected with either control vector, Mcl-1 or Bcl-x$_L$ expression vectors (kindly provided by C Borner and U Maurer, University of Freiburg, Germany), or the combination thereof, using the Amaxa nucleofection method.

Statistical analysis. Differences between data sets were analyzed by unpaired Students *t*-test. *P*-values < 0.05 were considered statistically significant.

Conflict of interest

The authors declare no conflict of interest.

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