ROS-dependent phosphorylation of Bax by wortmannin sensitizes melanoma cells for TRAIL-induced apoptosis

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The pathways of reactive oxygen species (ROS)-mediated apoptosis induction, of Bax activation and the sensitization of tumor cells for TRAIL (TNF-related apoptosis-inducing ligand)-induced apoptosis are still largely elusive. Here, sensitization of melanoma cells for TRAIL by the PI3-kinase inhibitor wortmannin correlated to the activation of mitochondrial apoptosis pathways. Apoptosis was dependent on Bax and abrogated by Bcl-2 overexpression. The synergistic enhancement was explained by Bax activation through wortmannin, which tightly correlated to the characteristic Bax phosphorylation patterns. Thus, wortmannin resulted in early reduction of the Bax-inactivating phosphorylation at serine-184, whereas the Bax-activating phosphorylation at threonine-167 was enhanced. Proving the responsibility of the pathway, comparable effects were obtained with an Akt inhibitor (MK-2206); while suppressed phosphorylation of serine-184 may be attributed to reduced Akt activity itself, the causes of enhanced threonine-167 phosphorylation were addressed here. Characteristically, production of ROS was seen early in response to wortmannin and MK-2206. Providing the link between ROS and Bax, we show that abrogated ROS production by α-tocopherol or by NADPH oxidase 4 (NOX4) siRNA suppressed apoptosis and Bax activation. This correlated with reduced Bax phosphorylation at threonine-167. The data unraveled a mechanism by which NOX4-dependent ROS production controls apoptosis via Bax phosphorylation. The pathway may be considered for proapoptotic, anticancer strategies.

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Induction of apoptosis is explained by two major signaling pathways. Whereas extrinsic pathways are initiated by death ligands, intrinsic pathways are induced by cellular and DNA damage. On one hand, clustering of death receptors in death-inducing signaling complexes leads to the activation of initiator caspase-8 and -10; on the other hand, mitochondria are employed leading to depolarization of the mitochondrial membrane potential (∆ψm) and release of cytochrome c, Smac and AIF.¹⁻³ Cytochrome c may induce activation of initiator caspase-9, and the different initiator caspases cleave and activate the executor caspases (-3, -6 and -7), which irreversibly trigger apoptosis by cleavage of a multitude of death substrates.⁴

Mitochondrial apoptosis pathways are critically controlled by Bcl-2 proteins, enclosing antiapoptotic (e.g. Bcl-2 and Mcl-1), proapoptotic multidomain (e.g. Bax and Bak) and proapoptotic BH3-only proteins (e.g. Puma, Noxa and Bid).⁵ The extrinsic pathway may also be enhanced via the mitochondria, as caspase-8 can process and activate the proapoptotic BH3-only protein Bid leading to truncated Bid (tBid).⁵

Bax plays a decisive role in death ligand-induced apoptosis.⁶ Its activation correlates with its mitochondrial translocation and with the conformational changes at the N-terminus (Bax-NT).⁷ The question how Bax activation is regulated, however, remained largely elusive, and only phosphorylation was discussed.⁸⁻¹⁰ Phosphorylation at serine-184 was suggested as inactivating¹⁰,¹¹ and phosphorylation at threonine-167 as an activating step.¹²⁻¹³ Also, reactive oxygen species (ROS) may contribute to the regulation of apoptosis; however, the relation to the described apoptosis pathways again remained elusive. ROS may be produced by NADPH oxidases.¹⁴⁻¹⁶

TRAIL (TNF-related apoptosis-inducing ligand) induces apoptosis via death receptors DR4 and DR5. It represents a promising anticancer strategy, due to the selective targeting of cancer cells.¹⁷,¹⁸ However, only limited efficacy was seen in clinical trials, which appeared as being related to inducible TRAIL resistance.¹⁹,²⁰ Treatment with TRAIL was also discussed for therapy-resistant melanoma, but again inducible resistance was seen.²¹,²²

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Abbreviations: AIF, apoptosis-inducing factor; ASK-1, apoptosis signal-regulating kinase 1; CDKs, cell cycle-dependent kinases; ∆ψm, mitochondrial membrane potential; ERK, extracellular signal-regulated protein kinases; GAPDH, glucose-6-phosphate dehydrogenase; JNK, c-Jun N-terminal kinase; LDH, lactate dehydrogenase; NOX4, NADPH oxidase 4; PI3K, phosphoinositol 3-kinase; PKC, protein kinase C; ROS, reactive oxygen species; Smac, second mitochondrial derived activator of caspases; tBid, truncated Bid; TRAIL, TNF-related apoptosis-inducing ligand; TS, TRAIL-selected; VDAC, voltage-dependent anion channel; XIAP, X-chromosome-linked inhibitor of apoptosis protein

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Protein kinase inhibitors are attracting increasing attention for cancer therapy since the last few years, and BRAF inhibitors recently led to a breakthrough in melanoma therapy.23 Besides MAP kinases, the pathway via phosphoinositide 3-kinases (PI3K) and protein kinase B (Akt) promotes proliferation and survival of tumor cells,24 as also reported for melanoma.25,26 For targeting PI3K, the fungal metabolite wortmannin has been identified as a specific and covalent inhibitor.27 Here, we show enhancement of TRAIL-induced apoptosis by wortmannin, and the underlying mechanisms are also analyzed. Thereby, we describe a new pathway, which provides a link between ROS and Bax activation.

Results

Enhanced TRAIL-induced apoptosis by wortmannin.

For overcoming melanoma resistance to TRAIL-induced apoptosis, the PI3K inhibitor wortmannin was evaluated in TRAIL-sensitive melanoma cell lines (A-375, Mel-HO), in cell lines that had been selected for TRAIL resistance (A-375-TS, Mel-HO-TS) and in permanent TRAIL-resistant cell lines (MeWo, Mel-2a). TRAIL-selected cells (A-375-TS) revealed resistance even at increased concentrations of TRAIL and at threonine-308 after treatment with 4 \( \mu \)M wortmannin are shown by western blotting in A-375 and A-375-TS. The signals of an antibody for total Akt are shown as controls. Equal protein loading (30 \( \mu \)g per lane) was proven by GAPDH. (c) Apoptosis in response to wortmannin (4 \( \mu \)M, 8 \( \mu \)M) – TRAIL (20 ng/ml) was monitored by cell cycle analysis in six melanoma cell lines (A-375, A-375-TS, Mel-HO, Mel-HO-TS, MeWo and Mel-2a). Cytotoxicity was determined in parallel by LDH release (in %). Statistical significance (* \( P < 0.005 \)) is indicated when comparing TRAIL-treated and wortmannin/TRAIL-treated cells. Insets: Histogram examples of cells treated with wortmannin/TRAIL (open graphs) as compared to DMSO controls (gray). Sub-G1 cell populations are indicated (sG1). (d) Real-time growth curves of A-375 and A-375-TS treated with wortmannin (4 \( \mu \)M) and/or TRAIL (20 ng/ml) were compared to DMSO controls. Cell indices were normalized at the time of treatment (24 h after seeding).

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Apoptosis was analyzed by cell cycle analyses, and apoptotic cells were clearly identified as cells with fragmented DNA (sub-G1 cells, Figure 1c, insets). Cytotoxicity was determined in parallel by LDH release (in %). Statistical significance (* \( P < 0.005 \)) is indicated when comparing TRAIL-treated and wortmannin/TRAIL-treated cells. Insets: Histogram examples of cells treated with wortmannin/TRAIL (open graphs) as compared to DMSO controls (gray). Sub-G1 cell populations are indicated (sG1). (d) Real-time growth curves of A-375 and A-375-TS treated with wortmannin (4 \( \mu \)M) and/or TRAIL (20 ng/ml) were compared to DMSO controls. Cell indices were normalized at the time of treatment (24 h after seeding).
combinations. Thus, up to 28% (A-375) and 19% (Mel-HO) apoptosis were obtained in sensitive cells when treated with TRAIL and 4–8 μM wortmannin. In TRAIL-selected A-375-TS and Mel-HO-TS, apoptosis induction was up to 19% and 13%, respectively. Only the permanent resistant cell lines MeWo and Mel-2a remained largely resistant (<7%). At 24 h, cytotoxicity (LDH release) played no role in the responsive cells (Figure 1c). Induced apoptosis by the combinations resulted in a significant decrease of cell proliferation, as determined by real-time cell analysis in A-375 and A-375-TS (Figure 1d).

No caspase activation but enhanced ROS. For understanding the pathways of wortmannin/TRAIL-induced apoptosis, activation of caspases and of Bid was investigated by western blotting at 8 h of treatment. Despite limited/lacking apoptosis induction in A-375/A-375-TS by TRAIL alone, the initiator caspase-8 was activated, evident by its cleavage products of 43, 41 and 18 kDa. Also, Bid was processed, evident by the reduction of its proform (22 kDa). However, the main effector caspase-3 appeared as largely arrested at a premature 20-kDa cleavage product; and caspase-9 was not cleaved. Paradoxically, caspase and Bid processing were not enforced by the combination (Figure 2a), despite enhanced apoptosis. In agreement with the caspase-independent pathways, apoptosis by TRAIL/wortmannin was only partially prevented by the pancaspase inhibitor Q-VD-OPh (10 μM), as shown in A-375 (30%→20%) and Mel-HO (22%→17%).

Figure 2 No enhanced caspase processing but ROS production by wortmannin/TRAIL. (a) Processing of caspase-8, -9, -3 and cleavage of Bid was monitored by western blotting in A-375 and A-375-TS in response to 8 h of treatment with wortmannin (4 μM) and/or TRAIL (20 ng/ml). Two independent experiments revealed highly comparable results. (b) Apoptosis (% of sub-G1 cells) is shown for A-375 and Mel-HO treated with TRAIL (20 ng/ml) and/or wortmannin (4 μM) –/+ the pancaspase inhibitor Q-VD-OPh (10 μM, 1 h pretreatment). Cytotoxicity was determined in parallel by a LDH release assay (in %). (c) ROS levels (10 000 cells gated) were determined by H2DCFDA staining and flow cytometry in A-375 and A-375-TS at 1, 2, 4 and 8 h after treatment with 20 ng/ml TRAIL, 4 μM wortmannin and/or 200 μM α-tocopherol (1 h pretreatment). Treated cells (open graphs) were compared to DMSO controls (gray). Higher fluorescence corresponds to increased ROS levels. (d) Apoptotic rates (% of sub-G1 cells) were determined in A-375 and A-375-TS in response to TRAIL (20 ng/ml), wortmannin (4 μM) and/or α-tocopherol (200 μM, 1 h pretreatment).
In contrast, apoptosis induced by TRAIL alone was completely blocked (Figure 2b). Thus, mechanisms were suggestive that induced apoptosis caspase-independently.

Caspase-independent apoptosis in melanoma cells can result from enhanced levels of ROS, as we have previously shown.\(^\text{15}\) Indeed, ROS levels were significantly increased in A-375 and A-375-TS by wortmannin alone at 1 h, which further increased with time. In contrast, TRAIL alone had no significant effect on ROS (Figure 2c). The critical role of ROS in apoptosis induction by wortmannin/TRAIL became evident by the ROS scavenger α-tocopherol (vitamine E). Pretreatment for 1 h with α-tocopherol prevented ROS production by wortmannin (Figure 2c) and reduced wortmannin/TRAIL-induced apoptosis by about 50% (Figure 2d).

**Activation of mitochondrial proapoptotic pathways.** In parallel with ROS, activation of proapoptotic mitochondrial pathways was seen. Thus, the \(\Delta\psi_m\) was decreased by wortmannin alone and in combination at early times (1 h), suggesting loss of \(\Delta\psi_m\) as a regulatory step and not as a result of induced apoptosis. TRAIL alone resulted in a slight decrease of \(\Delta\psi_m\) only in A-375 but not in A-375-TS, and TRAIL did not enhance the effect of wortmannin on \(\Delta\psi_m\). Loss of \(\Delta\psi_m\) by wortmannin was abrogated by α-tocopherol, thus proving a tight relation between ROS and the mitochondrial response (Figure 3a).

Combination treatments resulted in the release of cytochrome c, Smac and AIF, as determined at 2 h, which was also strongly diminished by α-tocopherol, thus proving a tight relation between ROS and the mitochondrial response (Figure 3a).

**Figure 3** Response of mitochondria and Bcl-2 proteins. (a) Decreased mitochondrial \(\Delta\psi_m\) was determined by flow cytometry after TMRM \(^{+}\) staining in A-375 and A-375-TS. Cells were treated for 1–8 h with TRAIL (20 ng/ml), wortmannin (4 \(\mu\)M) and/or α-tocopherol (200 \(\mu\)M, 1 h pretreatment). Treated cells (open graphs) were compared to DMSO controls (gray). Three independent experiments (each with triplicate values) revealed comparable results. (b) Cytosolic extracts (Cyto) of A-375 and A-375-TS, treated for 2 h with wortmannin, TRAIL and/or α-tocopherol, were analyzed by western blotting for the release of cytochrome c, Smac and AIF. Equal protein amounts were loaded as proven by incubation with GAPDH. A mitochondrial extract (Mito) served as control. A western blot for VDAC (50 kDa) ruled out contaminations of cytosolic extracts with mitochondria. A protein band of 37 kDa seen for A-375 traces back to a previous incubation of the blot with GAPDH (c) Expression of Bcl-2 proteins was determined by western blotting in A-375 and A-375-TS in response to wortmannin (4 \(\mu\)M) and/or TRAIL. Some slight differences seen for some proteins were not reproducible in the second, independent experiment (data not shown).
Decisive roles of Bcl-2 and Bax. Despite no change at the expression level, decisive roles were assigned to Bcl-2 and Bax. Thus, wortmannin/TRAIL-induced apoptosis was completely prevented by Bcl-2 overexpression in the stably transfected A-375 cells (A-375-Bcl-2), whereas the mock-transfected cells (A-375-pIRES) were sensitive (Figure 4a). Knockdown of Bcl-2 by siRNA, interestingly, enhanced apoptosis induction by wortmannin alone in A-375 and Mel-HO (15%, 17%). However, apoptosis by TRAIL/wortmannin was only slightly enhanced (A-375: 38% - 46%; Mel-HO: 21% - 29%), which may be attributed to the enhanced direct effects of wortmannin. Cytotoxicity (LDH release) remained unaffected (Figure 4b).

These data suggested that overexpressed Bcl-2 abrogated the contribution of TRAIL in the combination. On the other hand, Bcl-2 downregulation was redundant to the TRAIL effect, as Bcl-2 could also be blocked by TRAIL-induced Bid activation. Also, loss of Δψm in response to wortmannin/TRAIL was abrogated by Bcl-2 overexpression (Figure 4c). However, ROS production in response to wortmannin remained unaffected from Bcl-2 and was similarly blocked by α-tocopherol in both A-375-pIRES and A-375-Bcl-2 (Figure 4d). Thus, ROS production appeared to be independent of the mitochondrial response.

The roles of Bax and Bak were distinguished in a HCT-116 cell culture model that consisted of Bax+/Bak+/parental, Bax knockout, Bak knockdown and double knockdown cells. TRAIL/wortmannin significantly induced apoptosis at 24 h in parental and Bak knockdown cells, demonstrating that Bak played no role in this setting. In clear contrast, Bax knockout completely abrogated TRAIL- and TRAIL/wortmannin-induced apoptosis (Figure 5a). Also, in A-375 and Mel-HO melanoma cells, Bax knockdown by siRNA abrogated TRAIL- and TRAIL/wortmannin-induced apoptosis. Again, cytotoxicity played no role (Figure 5b).

Further following the pathway, loss of Δψm in response to wortmannin or TRAIL/wortmannin was also seen in HCT-116 parental and Bax knockdown cells, whereas Bak knockout and Bax/Bak double knockdown abolished the effects on Δψm (Figure 5c). In contrast, ROS production was independent of Bax/Bak expression (Figure 5d), thus suggesting ROS as initial and upstream of mitochondria.

The essential role of Bax for the mitochondrial response was further investigated by Bax siRNA. Thus, loss of Δψm in response to wortmannin and wortmannin/TRAIL was strongly diminished (A-375) or blocked (A-375-TS, Mel-HO) when cells were transfected with Bax siRNA. In contrast, control
RNA-transfected cells revealed a regular loss of $\Delta \psi m$ at 2 h of treatment (Figure 5e).

The critical role of Bax became more evident by its activation. Thus, increased Bax mitochondrial translocation was seen in A-375 and in A-375-TS already at 2 h (Figure 5f). The time dependency was investigated by the Bax-NT antibody, which selectively binds to the activated Bax with accessible N-terminus.\(^{28}\) Significant Bax activation appeared already at 2 h in A-375 and A-375-TS. Of note, activated Bax was not a result of induced apoptosis, but was seen earlier and in response to wortmannin treatment alone (Figure 5g). Bax activation by wortmannin was prevented by Bcl-2 overexpression (A-375-Bcl-2; Figure 5h), proving Bax suppression by Bcl-2 in wortmannin/TRAIL-induced apoptosis.
Bax activation by wortmannin through altered phosphorylation. Addressing the relation between kinase inhibition and Bax activation, assays were applied for monitoring Bax phosphorylation at serine-184 and threonine-167. Assays were based on cell permeabilization, staining with phosphorylation-specific antibodies and flow cytometry. Bax phosphorylation at serine-184 had been described as an inactivating step, while phosphorylation at threonine-167 was described as an activating step. Reliability of the used antibodies was checked in the HCT-116 Bax knockout cells. Whereas Bax antibodies resulted in significant signals in the HCT-116 parental cells, Bax knockout cells revealed no staining, thus excluding non-specific binding (Figure 6a).

At the time, when wortmannin resulted in Bax activation (Bax-NT, 2 h), it also revealed characteristic changes in Bax phosphorylation. Thus, phosphorylation at the deactivating serine-184 site was decreased, while phosphorylation at the activating threonine-167 site was enhanced. In contrast, treatment with TRAIL revealed no effect on Bax phosphorylation (Figure 6b and c). These data suggested that wortmannin reprogrammed melanoma cells for enhanced apoptosis sensitivity, although it did not trigger apoptosis by itself. The switch appeared to be altered Bax phosphorylation.

Unraveling the role of ROS in this setting, cells were treated with α-tocopherol. The antioxidant itself remained without effect on Bax phosphorylation, but reduced the enhanced threonine-167 phosphorylation in response to wortmannin (Figure 6c). In contrast, reduced phosphorylation at serine-184 remained unaffected by α-tocopherol (Figure 6b), thus distinguishing the regulation of the two Bax phosphorylation sites. Only threonine-167 appeared as dependent on ROS. Reduced threonine-167 phosphorylation correlated with reduced Bax activation, as seen by the Bax-NT antibody staining (Figure 6d), while no changes were seen in the Bax total protein level (Figure 6e).

Parallel effects of an Akt inhibitor on apoptosis and Bax. For addressing the relation of PI3K and Akt inhibition in this setting, some experiments were repeated with the allosteric Akt inhibitor MK-2206. It resulted in a concentration-dependent and long-lasting (24 h) downregulation of Akt phosphorylation, as shown for serine-473 in A-375, A-375-TS and Mel-HO (Figure 7a). Comparable to wortmannin, MK-2206 resulted in a significant and dose-dependent enhancement of TRAIL-induced apoptosis in TRAIL-sensitive and in resistant melanoma cells, as determined at 24 h by cell cycle analyses (A-375, 48%; Mel-HO, 24%; A-375-TS, 28%). Again, cytotoxicity played no role (Figure 7b). In parallel to wortmannin, ROS levels were significantly enhanced by MK-2206, as shown in A-375 and Mel-HO at 2 h of treatment, and ROS production was interrupted by α-tocopherol (Figure 7c).

Also, MK-2206 resulted in characteristic changes in Bax phosphorylation, as decreased serine-184 and enhanced threonine-167 phosphorylation, seen in A-375, A-375-TS and Mel-HO at 2 h of treatment. Furthermore, changes correlated with an enhanced Bax activation (Bax-NT; Figure 7d).

NOX4 is essential for wortmannin-induced ROS production. For unraveling the ROS pathway in this setting, the function of NADPH oxidase 4 (NOX4) was investigated. Downregulation of NOX4 by siRNA significantly reduced apoptosis by wortmannin/TRAIL, as shown in A-375 (44% → 19%) and Mel-HO (28% → 11%). In contrast, TRAIL-induced apoptosis in A-375 was not affected by NOX4 siRNA (Figure 8a). Proving the critical role of NOX4 for wortmannin-induced ROS, NOX4 siRNA completely suppressed ROS production in A-375 and Mel-HO (Figure 8b).

Highly comparable to the effects of α-tocopherol on Bax phosphorylation and Bax activation, displayed in Figure 6, also downregulation of NOX4 by siRNA completely abrogated the enhanced phosphorylation at threonine-167, but remained without effect on serine-184, as shown in A-375, A-375-TS and Mel-HO at 2 h of treatment. Abrogation of wortmannin-mediated Bax phosphorylation at threonine-167 by NOX4 knockdown also suppressed Bax activation (Bax-NT, Figure 8c). These data suggest a NOX4-dependent pathway leading from PI3K inhibition to Bax activation (Figure 8d).

Discussion

Based on the above data, a new pathway is suggested that depends on ROS-mediated Bax activation, and explains the sensitization of melanoma cells for TRAIL by wortmannin. As shown here as well as in previous work, TRAIL itself results in caspase-8 and Bid activation in both sensitive and resistant cells. Activated Bid can antagonize Bcl-2, and may thus overcome the Bcl-2-mediated antiapoptotic barrier in melanoma cells. However, a second antiapoptotic barrier is based
on the inactive Bax. This second barrier could be overcome by the PI3K inhibitor wortmannin, as demonstrated here (Figure 8d). The inhibitory effect of wortmannin on PI3K has been well described in previous studies resulting in suppression of the Akt prosurvival pathway. Furthermore, a role of Akt in
TRAIL resistance has already been suggested in different tumor cell models, including melanoma cells. As phosphorylation of Akt at serine-473 and threonine-308 represents a characteristic activity of PI3K, the here-seen downregulation of both phosphorylation sites in response to wortmannin clearly proved its specific inhibitory effect in melanoma cells. The role of the Akt pathway in the effects of wortmannin was furthermore proven by the allosteric Akt inhibitor MK-2206, which produced largely parallel effects to wortmannin in melanoma cells.

As concerns apoptosis regulation, phosphorylation of Bax at serine-184 by Akt has been reported, which was further related to an inhibition of Bax activity. Indeed, by applying a new phosphorylation assay, significant decrease of Bax phosphorylation at serine-184 is demonstrated here in response to wortmannin. But this de-phosphorylation obviously reflected only one half of the pathways supporting Bax activation. Thus, multiple pathways have been described downstream of Akt, which also include the regulation of two NADPH oxidases (NOX1, NOX4).

Figure 7  Enhanced TRAIL-induced apoptosis and Bax activation by the Akt inhibitor MK-2206. (a) A-375, A-375-TS and Mel-HO cells were treated with increasing concentrations of the allosteric Akt inhibitor MK-2206 (0.5–8 μM), and the effects on Akt phosphorylation (serine-473) were monitored by western blotting after 24 h. Signals of an antibody for total Akt (Akt) served as controls. (b) Apoptosis (percentage of sub-G1 cells) and cytotoxicity (LDH release) were determined in these cells after 24 h of treatment (means and SDs of two independent experiments, each one with triplicates). Statistical significance (*P < 0.005) is indicated, when comparing treatments with MK-2206/TRAIL and TRAIL alone. (c) ROS levels were determined by H$_2$DCFDA staining and flow cytometry in A-375 and Mel-HO at 2 h after starting treatment with 20 ng/ml TRAIL, 4 μM MK-2206 and/or α-tocopherol (200 μM, 1 h pretreatment). Treated cells (open graphs) were compared to DMSO controls (gray). Two independent experiments (each with triplicates) revealed comparable results. (d) Flow cytometry signals for Bax phosphorylation (Ser-184; Thr-167) and Bax activation (Bax-NT) were compared in A-375, A-375-TS and Mel-HO cells after 2 h treatment with TRAIL (20 ng/ml), MK-2206 (4 μM) and MK-2206/TRAIL. Treated cells (open graphs) are shown in overlays with DMSO controls (gray). Two complete and independent experiments with triplicates revealed the same results.
Thus, enhanced NOX activity was reported in response to Akt inhibition in pancreatic carcinoma and in chronic myeloid leukemia cells.\textsuperscript{38,39} NOX enzymes may produce large amounts of superoxide, and are thus tightly related to cellular ROS pathways.\textsuperscript{40} Supporting the role of ROS-dependent pathways, we found...
significantly increased ROS levels in melanoma cells in response to wortmannin treatment. Of note, ROS production appeared as an early step already at 1 h, which it was also seen in response to wortmannin alone and clearly distinguishes this effect from secondary effects in course of induced apoptosis. The significance of ROS for efficient induction of apoptosis in melanoma cells has already been reported in a previous study. Its significance in wortmannin/TRAIL-induced apoptosis was proven here by the ROS scavenger α-tocopherol. The effect could be further narrowed down to the activity of NOX4, as its siRNA-mediated downregulation prevented wortmannin/TRAIL-mediated ROS production as well as induced apoptosis.

The way in which ROS could contribute to apoptosis became apparent by analyzing the Bax phosphorylation sites. In addition to decreased Bax phosphorylation at serine-184, which may be attributed to Akt kinase activity itself, wortmannin also enhanced Bax phosphorylation at threonine-167. Phosphorylation at this site has been previously suggested as a Bax-activating step and has been related to JNK and p38 pathways in staurosporine-treated hepatoma or chemotherapeutics-treated retinoblastoma cells. Whereas de-phosphorylation at threonine-168 appeared to be independent of ROS, threonine-167 phosphorylation was largely prevented by α-tocopherol as well as by NOX4 siRNA, proving its clear dependency on ROS. Other relations between ROS and Bcl-2 proteins were so far mainly based on the expression level. Thus, Bcl-2 and Bcl-xL were downregulated, and Bax and Bak were upregulated in squamous cell carcinoma cells as a delayed result of enhanced intracellular ROS levels. The here identified link between ROS and Bax phosphorylation can, however, also explain the early effects of ROS in apoptosis control. Several different kinases might be involved in the threonine-167 phosphorylation of Bax. Thus, activation of ASK-1, JNK, p38, ERK1/2, PKC and CDKs by ROS had been reported. The Bax phosphorylation and activation assays, shown here, proved that Bax can be regulated independently by the two phosphorylation sites (Thr-167 and Ser-184), and only the threonine-167 phosphorylation depended on ROS.

Bax is a master regulator in mitochondrial proapoptotic pathways, and its particular role in TRAIL-induced apoptosis has been unraveled previously. Also, TRAIL/wortmannin-induced apoptosis in melanoma cells was dependent on Bax, whereas the sister protein Bak played no role in this setting. Thus, Bak activity in melanoma cells had been reported as being controlled by distinct pathways related to VDAC2. Bcl-2 represents the most critical antiapoptotic factor in melanoma. The model of mutual regulation of Bcl-2 proteins by heterodimerization, in which Bcl-2 acts as a particular antagonist of Bax, explains the strong antiapoptotic potential of Bcl-2 seen here. Thus, ectopic overexpression of Bcl-2 completely abrogated wortmannin/TRAIL-induced apoptosis as well as the loss of ΔΨm and the activation of Bax (Bax-NT).

The synergistic enhancement of apoptosis by wortmannin and TRAIL is further explained by the proapoptotic Bcl-2 protein Bid, which is cleaved and activated by caspase-8,7 Thus, Bid activation by TRAIL in TRAIL-sensitive A-375 and TRAIL-resistant A-375-TS melanoma cells is shown here and was also previously reported by us. Furthermore, Bid knockdown prevented TRAIL-induced apoptosis in other combinations. Truncated Bid acts as an efficient antagonist, particularly of Bcl-2. Thus, the basal Bcl-2 levels in melanoma cells appeared sufficient to prevent apoptosis by wortmannin alone, despite the activation of Bax. This was also proven by siRNA knockdown of Bcl-2, which enabled apoptosis induction by wortmannin alone. On the other hand, efficient apoptosis by TRAIL alone was prevented by the inactivated Bax. The breakthrough in the combination treatment therefore resulted from Bax activation by wortmannin and simultaneous Bcl-2 inactivation by TRAIL-induced tBid (Figure 8d).

As concerns the downstream pathways in wortmannin/TRAIL-induced apoptosis, significant release of apoptogenic mitochondrial factors (cytochrome c, Smac and AIF) was seen. The well-described pathways via cytochrome c and Smac should induce caspase activation, which was however not seen here. Also, other, independent strategies for directly increasing ROS levels in melanoma cells have shown efficient apoptosis induction (DNA fragmentation), while caspases were completely shut off. This discrepancy may be explained by an inhibitory activity of ROS on caspases. Thus, ROS-mediated thiol oxidation may also result in the oxidization of the cysteine residues in the caspase catalytic sites, thus abrogating caspase activity and procaspase processing. The efficient apoptosis induction seen in the present study, however, proves the existence of strong pathways in melanoma cells, which drive DNA fragmentation independently of caspases. Under these conditions, cytochrome c and Smac should lead to a dead end for apoptosis induction and other mitochondrial factors may take over, such as AIF and endonuclease G, which can trigger apoptosis independently of caspases.

The present data suggest that melanoma apoptosis resistance is based on several levels, including high Bcl-2 as reported previously as well as Bax inactivated by phosphorylation/de-phosphorylation. A third level was described recently, which is based on high XIAP expression, which prevents caspase activation and may be overcome by release of Smac. The inhibition by XIAP, however, was
of no role in the present setting, as circumvented by a ROS-dependent and caspase-independent pathway.

The death ligand TRAIL has been suggested as a promising antitumor strategy, although inducible resistance was seen in different tumor models, also including melanoma.\(^6\) Inducible resistance may also be the cause for the limited efficacy seen so far in clinical trials.\(^7\) Providing some hope, several strategies have been published in recent times that show efficient sensitization for TRAIL-induced apoptosis.\(^8\)

**Materials and Methods**

**Cell culture.** Human melanoma cell lines enclosed TRAIL-sensitive (A-375, Mel-HO) and TRAIL-resistant cells (Mel-2a and MeWo).\(^9\) Sublines with induced TRAIL resistance (Mel-HO-TS, A-375-TS) were derived from a selection with 100 ng/ml TRAIL.\(^2\) Other A-375 subclones had been stably transfected with a pIREs-Bcl-2 plasmid for Bcl-2 overexpression (A-375-Bcl-2) or with the pIREs empty plasmid (A-375-pIREs), as described previously.\(^7\) Parental HCT-116 colon carcinoma cells were derived from ATCC (Maryland, MD, USA). The HCT-116 Bax knockout, Bax knockdown and Bax/Bak double knockdown cells were kindly provided by B Vogelstein (John Hopkins Cancer Center, Baltimore, Maryland, MD, USA) and had been described previously.\(^6\)

Cells were cultivated at 37 °C, 5% CO2 in DMEM (4.5 g/l glucose; Gibco, Invitrogen, Karlsruhe, Germany), and supplemented with 10% FCS and antibiotics (Biochrom, Berlin, Germany). TRAIL-selected cells were continuously kept with 5 ng/ml TRAIL until 24 h before treatment. Cells were plated in 6-, 24- or 96-well plates with 2 × 10^4, 5 × 10^4 and 5 × 10^5 cells, respectively, and treatment was started after 24 h. For induction of apoptosis, TRAIL (Alexis, Gruenberg, Germany, ALX-201-073-C020, 20 ng/ml), the PI3K inhibitor wortmannin (4–8 μM of the pancaspase inhibitor Q-VD-OPh (MP Biomedicals, Solon, OH, USA) and had been described previously.\(^6\)

**Cell proliferation, apoptosis and cytotoxicity.** For continuous monitoring of cell growth, the xCELLigence system (Roche Diagnostics, Penzberg, Germany) was applied, and the relative cell indices were determined, which correspond to attached cell numbers. For quantification of apoptosis, cell cycle analyses were performed in PBS/1% FCS, which also contained 200 μM α-L-naphthylmalononitrile (Sigma-Aldrich, Taufkirchen, Germany; 200 mg/ml).

**Statistical analyses.** Assays consisted of triplicates, and at least two independent experiments were performed. Mean values and SDs were calculated by enclosing all individual values of the independent experiments (at least six values). Statistical significance was proven by Student’s t-test (normal distribution) or Wilcoxon test (non-normal distribution of data), and P-values of < 0.05 were considered statistically significant.

**Conflict of Interest**

The authors declare no conflict of interest.

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