Targeting Aberrant PI3K/Akt Activation by PI103 Restores Sensitivity to TRAIL-Induced Apoptosis in Neuroblastoma

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Abstract

Purpose: Because we recently identified Akt activation as a novel poor prognostic indicator in neuroblastoma, we investigated whether phosphoinositide 3’-kinase (PI3K) inhibition sensitizes neuroblastoma cells for TRAIL-induced apoptosis.

Experimental Design: The effect of pharmacological or genetic inhibition of PI3K or mTOR was analyzed on apoptosis induction, clonogenic survival, and activation of apoptosis signaling pathways in vitro and in a neuroblastoma in vivo model. The functional relevance of individual Bcl-2 family proteins was examined by knockdown or overexpression experiments.

Results: The PI3K inhibitor PI103 cooperates with TRAIL to synergistically induce apoptosis (combination index < 0.1), to suppress clonogenic survival, and to reduce tumor growth in a neuroblastoma in vivo model. Similarly, genetic silencing of PI3K significantly increases TRAIL-mediated apoptosis, whereas genetic or pharmacological blockage of mTOR fails to potentiate TRAIL-induced apoptosis. Combined treatment with PI103 and TRAIL enhances cleavage of Bid and the insertion of tBid into mitochondrial membranes, and reduces phosphorylation of BimEL. Additionally, PI103 decreases expression of Mcl-1, XIAP, and cFLIP, thereby promoting Bax/Bak activation, mitochondrial perturbations, and caspase-dependent apoptosis. Knockdown of Bid or Noxa or overexpression of Bcl-2 rescues cells from PI103- and TRAIL-induced apoptosis, whereas Mcl-1 silencing potentiates apoptosis. Bcl-2 overexpression also inhibits cleavage of caspase-3, caspase-8, and Bid pointing to a mitochondria-driven feedback amplification loop.

Conclusions: PI103 primes neuroblastoma cells for TRAIL-induced apoptosis by shifting the balance toward proapoptotic Bcl-2 family members and increased mitochondrial apoptosis. Thus, PI3K inhibitors represent a novel promising approach to enhance the efficacy of TRAIL-based treatment protocols in neuroblastoma. Clin Cancer Res; 17(10); 3233–47. ©2011 AACR.

Introduction

Neuroblastoma represents the most common extracranial solid tumor outside the central nervous system in childhood (1, 2). The prognosis of children older than 1 year with stage 4 disease is still very poor with long-term survival rates of only 40% despite intensive and multimodal therapy (3). Therefore, novel strategies are required for the treatment of advanced stage neuroblastoma.

Programmed cell death or apoptosis is essential to maintain tissue homeostasis (4). In addition, induction of apoptosis in malignant cells presents a key principle of cancer therapy (5). There are 2 major pathways that initiate apoptosis and eventually lead to activation of caspasases: one is initiated by death receptors on the cell surface (death receptor or extrinsic pathway), whereas the other is mediated by the mitochondria (mitochondrial or intrinsic pathway; ref. 5). The death receptor pathway is typically stimulated by ligation of death receptors of the TNF receptor superfamily by their cognate ligands or agonistic antibodies, whereas the other is mediated by the mitochondria (mitochondrial or intrinsic pathway; ref. 5). The death receptor pathway is typically stimulated by ligation of death receptors of the TNF receptor superfamily by their cognate ligands or agonistic antibodies, resulting in the formation of the death-inducing signaling complex (DISC; ref. 6). This in turn leads to caspase-8 activation, which can directly cleave downstream caspases (6). Stimulation of the mitochondrial apoptosis pathway culminates in the permeabilization of the outer mitochondrial membrane, the release of cytochrome c into the cytosol, and caspase-3 activation via the apoptosome complex (7). Members of the Bcl-2 family proteins are critical regulators of apoptosis by controlling outer mitochondrial membrane permeabilization (8). Bcl-2 family proteins can be divided into antiapoptotic proteins, such as Bcl-2 and Mcl-1 or proapoptotic proteins like Bax, Bak, and BH3 domain-only molecules (e.g., Bid, Bim, and Noxa;
Translational Relevance

Recently, we identified aberrant Akt activation as a novel indicator of poor prognosis in neuroblastoma. In this study, we investigated whether the recently developed PI3K inhibitor PI103 decreases the threshold for TRAIL to trigger apoptosis in neuroblastoma. Here, we provide first evidence that PI103 primes neuroblastoma cells for TRAIL-induced apoptosis in a synergistic manner in vitro and also in an in vivo model by shifting the balance toward prosurvival Bcl-2 family members and activation of the mitochondrial apoptosis pathway. This preclinical evaluation of a rational combination of 2 novel classes of targeted drugs, that is, the PI3K inhibitor PI103, and TRAIL, identifies a novel indication for PI103 in TRAIL-based combination therapies. This provides the molecular basis for the design of future clinical studies in neuroblastoma and other cancers with aberrant PI3K/Akt activation and thus has important clinical implications.

Materials and Methods

Cell culture and chemicals

Neuroblastoma cell lines were obtained from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium (SH-EP), RPMI 1640 (LAN-5), or MEM (CHP-212) medium (Life Technologies, Inc.), supplemented with 10% fetal calf serum (Biochrom), 1 mmol/L glutamine (Biochrom), 1% penicillin/streptomycin (Biochrom), and 25 mmol/L HEPES (Biochrom) as described previously (14). N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk) was purchased from Bachem, PI103 from Alexis Biochemicals, LY294002 from Calbiochem, and TRAIL was obtained from R&D Systems. RAD001 (everolimus) was kindly provided by Novartis Institute for BioMedical Research (Oncology Basel, Novartis Pharma AG, Basel, Switzerland). All other chemicals were purchased by Sigma, unless otherwise indicated.

Determination of apoptosis

Apoptosis was determined by fluorescence-activated cell sorting (FACScan; BD Biosciences) analysis of DNA fragmentation of propidium iodide–stained nuclei as described previously (14). The percentage of specific apoptosis was calculated as follows: 100 × [experimental apoptosis (%) – spontaneous apoptosis (%)]/[100% – spontaneous apoptosis (%)].

Colony forming assay

To determine colony formation, 200 cells were seeded in a 6-well tissue culture plate and allowed to settle overnight. Cells were treated with TRAIL for 24 hours with or without 0.6 μmol/L PI103. Next day, medium was exchanged and colonies were stained after 10 days with 0.75% crystal violet, 50% ethanol, 0.25% NaCl, and 1.57% formaldehyde. Colonies were counted and the percentage of surviving colonies relative to solvent-treated controls was calculated.

Transient RNA interference

For transient gene knockdown cells were seeded, allowed to settle overnight and transfected with 60 pmol of PIK3CA Stealth RNAi (PIK3CAHS10800 4-6; Invitrogen), PIK3CB Stealth RNAi (PIK3CBHSS10800 7-9; Invitrogen), mTOR Stealth RNAi (FRAPIHSS10382 5-7), Stealth RNAi against Bax (BAXHSS14135 4-6; Invitrogen), Bak (BIDHS-S14147 6-8; Invitrogen), or Stealth RNAi nontargeting control (12935; Invitrogen) by using TransMessenger transfection (301525; Qiagen).

Transduction

For stable gene knockdown, short-hairpin RNA (shRNA)-targeting Noxa (5’-GATCCCGTATATATGACATTTTCTCAGAGAGAAATGTGTCAATAATTACTTTTGCTGGAGATTAT, GATTGTGACTCTCATTTCT) and a sequence with no corresponding part in the human genome (GATCATGTAGATACGCTCA) that was used as control were cloned into pRETSUPER and transduced with murine stem-cell virus (pMSCV) vector containing mouse Bcl-2 or empty vector using the packaging cell line P167 (BD Biosciences). Stable cell lines were selected by 10 μg/mL blasticidin (Invitrogen).
Western blot analysis

Western blot analysis was carried out as described previously (14) by using the following antibodies: rabbit anti–phospho-Akt (Ser473), rabbit anti–phospho-S6 ribosomal protein (Ser235/236), mouse anti-S6 ribosomal protein, rabbit anti-mTOR, rabbit anti–PI3K-p110α (1:1,000; Cell Signaling), mouse anti-Akt (1:500), mouse anti-XIAP (1:1,000; clone 28) and rabbit anti–Bcl-X L (1:1,000; BD Transduction Laboratories), rabbit anti-survivin (1:1,000; R&D Systems), mouse anti–caspase-8 (1:1,000; Alexis Biochemicals), mouse anti–caspase-3, rabbit anti-Bid and rabbit anti-Bim (1:1,000; Cell Signaling), rabbit anti–caspase-9, mouse anti–Bcl-2, rabbit anti-Bak, mouse anti–cytochrome c (1:1,000; BD Pharmingen), mouse anti-Noxa (1:1,000; Alexis), rabbit anti–Mcl-1 (Stressgen), mouse anti–caspase-3, mouse anti–GAPDH (1:5,000; HyTest), or mouse anti–α-tubulin (1:3,000; Calbiochem) were used as loading controls followed by goat anti-mouse immunoglobulin G (IgG) or goat anti-rabbit IgG conjugated to horseradish peroxidase (1:5,000; Santa Cruz Biotechnology). Enhanced chemiluminescence was used for detection (Amersham).
Determination of mitochondrial membrane potential and cytochrome c release

To determine mitochondrial transmembrane potential cells were incubated with CMXRos (1 μmol/L; Molecular Probes) for 30 minutes at 37°C and immediately analyzed by flow cytometry.

Cytochrome c release was assessed by flow cytometry as previously described (17). To analyze cytochrome c and Bid in cytosolic and mitochondrial extracts by Western blot cells were harvested and washed with PBS. Cells were suspended in lysis buffer [2 mmol/L NaH₂PO₄, 16 mmol/L Na₂HPO₄, 150 mmol/L NaCl, 500 mmol/L sucrose, 1 mmol/L Dithiothreitol (DTT), Protease Inhibitor (Roche), and 0.5 mg/ml digitonin] for 3 minutes on ice. Unbroken cells, mitochondria, and nuclei were removed by centrifugation at 14,000 rpm for 1 minute at 4°C. The supernatant was collected as cytosolic fraction and the pellet was resuspended in lysis buffer [30 mmol/L Tris HCL, 150 mmol/L NaCl, 1% Triton X, 10% glycerol (Invitrogen), Protease Inhibitor (Roche), 2 mmol/L DTT, 500 μmol/L phenylmethylsulfonylfluoride] for 2 hours at 4°C, and centrifuged at 14,000 rpm for 20 minutes at 4°C. The supernatant was collected as mitochondrial fraction. Protein expression of cytochrome c or Bid were analyzed by Western blotting. OxPhos Complex IV, α-tubulin, and β-actin were used to control the purity and loading of the mitochondrial and cytosolic fractions.

Determination of Bax and Bak activation

Bax and Bak activation was determined by immunoprecipitation as previously described (18). Briefly, cells were lysed in CHAPS lysis buffer (10 mmol/L HEPES, pH 7.4; 150 mmol/L NaCl; 1% CHAPS). Protein (1 mg) was
Figure 2. Inhibition of PI3K, rather than of mTOR, sensitizes SH-EP neuroblastoma cells to TRAIL-induced apoptosis. A, knockdown of PI3K sensitizes SH-EP neuroblastoma cells to TRAIL-induced apoptosis. SH-EP cells were transfected with siRNA duplex oligonucleotides against p100α and p110β, mTOR, or nontargeting siRNA (control). Knockdown of p110α, p110β, and mTOR expression and protein expression levels and phosphorylation status of Akt and S6 ribosomal protein were analyzed by Western blotting at 72 hours after transfection. α-Tubulin served as loading control (left). Seventy-two hours after transfection, cells were treated with 2 ng/mL TRAIL for 24 hours. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide–stained nuclei and percentage of specific apoptosis is shown (right). Data represent mean ± SEM of 3 independent experiments carried out in triplicate (*, P < 0.05). B, inhibition of mTOR by everolimus and rapamycin. SH-EP cells were treated for 24 hours with 0.6 μmol/L PI103, DMSO as solvent, everolimus, or rapamycin at the indicated concentrations. Protein expression level and phosphorylation status of Akt and S6 ribosomal protein were analyzed by Western blotting. α-Tubulin served as loading control. C, inhibition of mTOR fails to enhance TRAIL-induced apoptosis. SH-EP cells were left untreated (med) or were treated for 24 hours with 10 nmol/L everolimus (dark gray bars), 10 nmol/L rapamycin (light gray bars), 0.6 μmol/L PI103 (black bar), or DMSO as solvent (white bars) in combination with TRAIL at the indicated concentrations. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide–stained nuclei and percentage of specific apoptosis is shown. Data represent mean ± SEM of at least 3 independent experiments carried out in triplicate. *, P < 0.05.
incubated with 2 μg mouse anti-Bax antibody (6A7; Sigma) or anti-Bak antibody (AB-1; Calbiochem) overnight at 4°C followed by addition of 10 μL Dynabeads Pan Mouse IgG (Dako), incubated for 2 hours at 4°C, washed with CHAPS lysis buffer, and were analyzed by Western blotting by using rabbit anti-BaxNT or rabbit anti-Bak (BD Pharminagen) antibody.

Chorioallantoic membrane assay

Chorioallantoic membrane (CAM) assay was done as described previously (17). Briefly, 2 × 10⁶ cells were resuspended in 10 μL serum-free medium and 10 μL Matrigel matrix (BD Biosciences) and implanted on the CAM of fertilized chicken eggs on day 8 of incubation, allowed for 48 hours to form tumors, and treated with 1 μmol/L PI103 and/or 10 ng/mL TRAIL for 2 days. Four days after seeding tumors were sampled with the surrounding CAM, fixed in 4% paraformaldehyde, paraffin embedded, cut in 5 μmol/Lsections, and analyzed by immunohistochemistry by using 1:1 hematoxylin and 0.5% eosin. Images were digitally recorded at a magnification of ×2 with an AX70 microscope (Olympus), tumor areas were analyzed with ImageJ digital imaging software.

Statistical analysis

Statistical significance was assessed by Student’s t Test (2-tailed distribution, 2-sample, unequal variance).

Results

Sensitization of neuroblastoma cells to TRAIL-induced apoptosis by the PI3K inhibitor PI103

To analyze the role of PI3K signaling in the regulation of TRAIL-induced apoptosis in neuroblastoma, we selected from a panel of human neuroblastoma cell lines SH-EP, CHP-212, and LAN-5 neuroblastoma cells, because they all express caspase-8, a key component of the death receptor pathway (data not shown). Initially, we investigated the optimal conditions at which the recently developed class I PI3K inhibitor PI103 (19) blocks PI3K-mediated signaling in these cells. Dose–response and kinetic analysis revealed that PI103 inhibits PI3K signaling at nanomolar concentrations rapidly and for a prolonged time up to 48 hours.
Importantly, PI103 significantly enhances TRAIL-induced apoptosis in a dose- and time-dependent manner (Fig. 1B and C). Calculation of combination index revealed a synergistic interaction of PI103 and TRAIL (Supplementary Fig. S1). Also, PI103 significantly reduced colony formation on TRAIL treatment (Fig. 1D), showing an effect on long-term survival. These data show that inhibition of PI3K by PI103 sensitizes neuroblastoma cells to TRAIL-induced apoptosis.

Inhibition of PI3K is superior to mTOR inhibition for sensitizing SH-EP neuroblastoma cells to TRAIL-induced apoptosis

As we previously found that activation of Akt, but not of mTOR correlates with poor prognosis in neuroblastoma, we asked whether mTOR signaling may differentially modulate apoptosis sensitivity compared with PI3K. To address this question, we blocked PI3K/Akt/mTOR signaling selectively at the level of PI3K versus mTOR by using RNA interference (RNAi). Control experiments confirmed that silencing of the PI3K subunits p110α and p110β or of mTOR resulted in selective downregulation of the respective target proteins and to decreased phosphorylation of Akt (in case of p110α/p110β knockdown) or of S6 ribosomal protein (in case of mTOR or p110α/p110β knockdown; Fig. 2A). As silencing of mTOR inhibits both mTORC1 and mTORC2 complexes, the increase in Akt phosphorylation in cells with mTOR knockdown (Fig. 2A) may be due to differential sensitivities of mTORC1 and mTORC2 complexes to incomplete mTOR inhibition. Interestingly, knockdown of p110α/p110β significantly increased TRAIL-induced apoptosis (Fig. 2A). The reduced efficacy of genetic versus pharmacological inhibition of PI3K to confer sensitization to TRAIL-induced apoptosis may be due to incomplete knockdown of p110α/p110β. By comparison, silencing of mTOR did not alter sensitivity.
toward TRAIL (Fig. 2A). Similarly, pharmacological inhibition of mTOR by everolimus and rapamycin failed to sensitize neuroblastoma cells for TRAIL-induced apoptosis, although both inhibitors suppressed phosphorylation of S6 ribosomal protein at nanomolar concentrations (Fig. 2B and C). Doxorubicin was used as positive control for everolimus-mediated enhancement of apoptosis in neuroblastoma cells (20). These findings show that pharmacological or genetic inhibition of PI3K is superior to mTOR inhibition to sensitize neuroblastoma cells to TRAIL-induced apoptosis.

**TRAIL and PI103 cooperate to trigger Bid activation and mitochondrial outer membrane permeabilization**

To gain insights into the molecular mechanisms mediating apoptosis sensitization by PI3K inhibition, we monitored caspase cleavage by Western blot analysis. Combined treatment of TRAIL and PI103 enhanced the cleavage of caspase-8, caspase-3, and caspase-9 as well as the conversion of Bid into tBid (Fig. 3A, top). To test the requirement of caspases, we used the broad-range caspase inhibitor zVAD.fmk. Apoptosis on treatment with TRAIL and PI103 was completely blocked in the presence of zVAD.fmk (Fig. 3A, bottom), showing that apoptosis was mediated by caspases.

As tBid links the death receptor to the mitochondrial pathway by translocating to mitochondrial membranes, we then analyzed tBid in the mitochondrial fraction. Interestingly, PI103 and TRAIL cooperated to cause accumulation of tBid at mitochondrial membranes (Fig. 3B). Silencing of Bid via siRNA significantly reduced apoptosis triggered by PI103 and TRAIL and also by treatment with TRAIL alone (Fig. 3C), showing the requirement of tBid for apoptosis induction. Furthermore, PI103 significantly enhanced TRAIL-induced loss of mitochondrial membrane potential (MMP) and cytochrome c release (Fig. 3D). Translocation of tBid to mitochondrial membranes, loss of MMP, and cytochrome c release were all blocked in the presence of zVAD.fmk (Fig. 3B, 3D), showing that they occur in a caspase-dependent manner. This set of experiments shows that PI103 sensitizes neuroblastoma cells to TRAIL-induced caspase activation, Bid cleavage, translocation of tBid to mitochondria, and caspase-dependent mitochondrial outer membrane permeabilization.

**PI3K inhibition shifts the balance toward proapoptotic Bcl-2 proteins**

Next, we monitored protein expression levels of key apoptosis regulators by Western blot analysis. Combination treatment of TRAIL and PI103 reduced protein levels of Mcl-1, XIAP, survivin, cFLIPL, and cFLIPS (Fig. 4A). As Mcl-1 and Noxa are markedly downregulated and upregulated, respectively, on treatment with PI103 and TRAIL (Fig. 4A), we next explored their functional relevance on TRAIL receptor ligation and TRAIL receptor surface expression. However, we found no detectable changes in the recruitment of the adaptor molecule Fas-associated protein with Death Domain (FADD) or caspase-8 to stimulated TRAIL receptors within the first 4 hours on addition of TRAIL or in surface expression of agonistic TRAIL receptors (Supplementary Fig. S4). As Mcl-1 and Noxa are markedly downregulated and upregulated, respectively, on treatment with PI103 and TRAIL (Fig. 4A), we next explored their functional relevance by using RNAi-mediated silencing. Knockdown of Noxa significantly reduced, whereas silencing of Mcl-1 significantly increased apoptosis on combination treatment with TRAIL and PI103 (Fig. 4B). Also, apoptosis induced by treatment with TRAIL alone was enhanced and reduced when Mcl-1 and Noxa were silenced, respectively (Fig. 4B). Also, knockdown of Mcl-1 led to increased PI103-mediated apoptosis (Fig. 4B).

As activation of Bax and Bak presents a central event in mitochondrial outer membrane permeabilization, we next analyzed their activation status by using conformation-specific antibodies. Notably, PI103 enhanced the TRAIL-mediated activation of Bax and Bak (Fig. 4C; Supplementary Fig. S5).
Supplementary Fig. S5). Simultaneous knockdown of Bax and Bak significantly reduced TRAIL- and PI103-mediated cell death (Fig. 4C), indicating that Bax and Bak are involved in PI103-confirmed sensitization to TRAIL-induced apoptosis. Together, this set of experiments indicates that changes in the expression or activation of pro- and antiapoptotic Bcl-2 proteins present critical molecular events for the PI103-mediated sensitization of neuroblastoma cells toward TRAIL.

**Overexpression of Bcl-2 prevents the synergistic induction of apoptosis by PI103 and TRAIL.**

To further investigate whether the mitochondrial pathway is required for the synergistic interaction of TRAIL and PI103, SH-EP cells were treated with 0.6 μmol/L PI103 or DMSO and/or 2 ng/mL TRAIL for indicated times. Protein expression of phospho-Akt, Akt, phospho-S6, S6, cFLIP, XIAP, Mcl-1, survivin, Bax, Bak, BimEL, and Noxa were determined by Western blotting. GAPDH and β-actin served as loading controls. The relative phosphorylation of Akt was densitometrically determined and is expressed in percentage of phospho-Akt/Akt ratio, with DMSO set as 100%. Changes of expression level of Mcl-1, Noxa, cFLIP, survivin, and XIAP are densitometrically determined and are expressed in percentage of protein/loading control ratio, with DMSO set as 100%.
PI103, we overexpressed Bcl-2 to interfere with mitochondrial perturbations. Ectopic expression of Bcl-2 profoundly reduced apoptosis on combination treatment with PI103 and TRAIL and also with TRAIL alone (Fig. 5A). In addition, Bcl-2 overexpression inhibited TRAIL-induced Bax activation, loss of MMP, as well as cleavage of caspase-8 into p43/p41 and p18 active fragments, cleavage of Bid, and processing of the caspase-3 p20 fragment into the p17/p12 active

![Graph showing specific apoptosis](image)

**Figure 4.** (Continued). B, SH-EP cells were stably transduced with a vector containing shRNA against Mcl-1, Noxa, or control shRNA by using pRETS-L-SUPER vector. Knockdown of Mcl-1 or Noxa expression was controlled by Western blotting; α-tubulin served as loading control (left). Transduced cells were treated with 0.6 μmol/L PI103 and/or 2 ng/mL TRAIL for 24 hours. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide–stained nuclei and percentage of specific apoptosis is shown. Data represent mean ± SEM of at least 2 independent experiments carried out in triplicate (†, P <0.001). C, SH-EP cells were treated with 0.6 μmol/L PI103 or DMSO with or without 2 ng/mL TRAIL for 4 hours. Bax- and Bak-activation were analyzed by immunoprecipitation of protein lysates by using active conformation-specific antibodies for Bax and Bak and Western blotting. D, SH-EP cells were transfected with siRNA duplex oligoribonucleotides against Bax and Bak (black bars) or with nontargeting control siRNA (white bars). Knockdown of Bax and Bak protein expression was assessed by Western blotting; α-tubulin was used as loading control (left). Seventy-two hours after transfection, cells were treated with 2 ng/mL TRAIL and/or 0.6 μmol/L PI103. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide–stained nuclei (right). Data represent mean ± SEM of 3 independent experiments carried out in triplicate (*, P < 0.05).
fragments (Fig. 5B–D). Likewise, inhibition of caspases by zVAD.fmk inhibited Bax activation (Fig. 5B) and Bid cleavage, its translocation to mitochondrial membranes, and loss of MMP (Fig. 3B and D). Together, these findings point to a mitochondria-controlled feedback amplification loop from caspase-3 to caspase-8 activation, Bid cleavage, and mitochondrial outer membrane permeabilization.

PI103 cooperates with TRAIL to suppress neuroblastoma growth in vivo

Finally, we investigated the antitumor activity of PI103 and TRAIL in vivo, using the CAM assay, an established in vivo tumor model for neuroblastoma (17, 18, 21, 22), for example. Neuroblastoma cells were seeded on the CAM of chicken embryos, allowed to settle and to initiate tumors, and then treated with TRAIL in the presence or absence of PI103. Importantly, PI103 and TRAIL acted in concert to significantly suppress tumor growth of neuroblastoma compared with either agent alone (Fig. 6). This shows that PI103 cooperates with TRAIL to suppress neuroblastoma growth in an in vivo model of neuroblastoma.

**Discussion**

We recently identified aberrant PI3K/Akt activation as a novel indicator of poor prognosis in neuroblastoma (13). Therefore, we investigated whether therapeutic targeting of PI3K/Akt by the recently developed PI3K inhibitor PI103 could restore apoptosis sensitivity in neuroblastoma. Here, we report for the first time that inhibition of PI3K is an efficient strategy to prime neuroblastoma cells to TRAIL-induced apoptosis. This conclusion is supported by several

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**Figure 5.** Bcl-2 overexpression prevents the synergistic induction of apoptosis by TRAIL and PI103.

A, SH-EP cells were transduced with mouse Bcl-2 (mBcl-2) or pMSCV empty vector (EV). Overexpression of mBcl-2 was assessed by Western blotting and α-tubulin served as loading control (left). Transduced cells were treated for 24 hours with 0.6 μmol/L PI103 and/or 5 ng/mL TRAIL (right). Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei. Data represent mean ± SEM of 2 independent experiments carried out in triplicate (*, P < 0.05, #, P < 0.001). B–D, transduced cells were treated for 4 hours with 0.6 μmol/L PI103, and/or 2 ng/mL TRAIL. B, Bax activation was analyzed by immunoprecipitation of protein lysates by using the active conformation-specific antibodies for Bax and Bak and Western blotting.
independent lines of evidence. First, pharmacological or genetic inhibition of PI3K synergistically enhances TRAIL-induced apoptosis as shown by calculation of combination index. Second, data obtained in several neuroblastoma cell lines underscore the generality of this finding. Third, the combination treatment of PI103 and TRAIL is superior to single agents in suppressing long-term survival in colony forming assays and cooperates to reduce tumor growth in an in vivo model of neuroblastoma, underlining the clinical relevance of our findings.

Mechanistically, our data show for the first time that PI3K inhibition acts in concert with TRAIL to cleave Bid into tBid and to trigger accumulation of tBid at mitochondrial membranes, an event that contributes to apoptosis induction as shown by knockdown experiments (Fig. 6B). Interestingly, PI103 enhances TRAIL-induced cleavage of caspase-8 and Bid in a mitochondrial feedback amplification loop, which likely involves caspase-3-mediated activation of caspase-8 and Bid, because Bcl-2 overexpression blocks the PI103-mediated enhancement of TRAIL-induced cleavage of caspase-3, caspase-8, and Bid. By comparison, PI103 has no effect on the recruitment of caspase-8 to the TRAIL DISC. This caspase-3–driven mitochondrial amplification loop is further facilitated by the PI103-mediated reduction of XIAP expression. To this end, Akt has been reported to protect XIAP from proteasomal degradation via phosphorylation (23).

Furthermore, we found that PI103 reduces phosphorylation of BimEL and Mcl-1 expression. The reduced phosphorylation of BimEL by PI103 is consistent with the Akt-mediated phosphorylation of BimEL, which enhances its proapoptotic degradation (24). Mcl-1 downregulation by PI103 may be the result of reduced Mcl-1 transcription on PI3K/Akt inhibition (25). In addition, the rapid downregulation of Mcl-1 points to PI103-mediated posttranslational events, for example, Mcl-1 degradation via inhibition of GSK3β phosphorylation on PI3K inhibition (26). In addition, Noxa exerts its proapoptotic function by antagonizing the antiapoptotic effect of Mcl-1 (27). The key involvement of Mcl-1 and Noxa in this PI103-mediated sensitization to TRAIL-induced apoptosis is supported by data showing that knockdown of Noxa rescues SH-EP neuroblastoma cells from apoptosis, whereas knockdown of Mcl-1 leads to increased cell death. These alterations in the expression or activation of tBid, Noxa, and Mcl-1 shift the balance toward proapoptotic Bcl-2 proteins, which enhances TRAIL-induced Bax activation and mitochondrial outer membrane permeabilization in the presence of PI103. Additionally, PI103 promotes Bax activation by preventing the Akt-mediated inhibitory phosphorylation of Bax (28, 29). The key role of the mitochondrial pathway is underscored by experiments carried out in Bcl-2 overexpressing cells, which are almost completely resistant to PI103- and TRAIL-induced apoptosis.

In contrast to PI3K inhibition, we found that genetic or pharmacological inhibition of mTOR does not confer sensitivity to TRAIL-induced apoptosis. This result is in accordance with our recent study showing that phosphorylation of S6 ribosomal protein does not correlate with poor prognosis in neuroblastoma, whereas phosphorylation of Akt does correlate (13). In glioblastoma, we similarly found that mTOR inhibition does not enhance TRAIL- or doxorubicin-induced apoptosis (20, 30). By comparison, mTOR inhibitors have been reported to suppress tumor growth and angiogenesis in neuroblastoma cells with MYCN amplification (31) and to induce apoptosis together with chemotherapeutic agents (20, 32), implying a context-dependent role of mTOR signaling in
neuroblastoma. In addition to neuroblastoma, PI3K inhibition has previously been reported to prime glioblastoma, leukemia, or colon carcinoma cells for TRAIL-induced apoptosis (20, 33–35).

Our findings have several important implications. First, our study translates our recent identification of PI3K/Akt as a potential therapeutic target in neuroblastoma (13) into the development of a novel strategy to prime neuroblastoma for TRAIL-induced apoptosis. Therapeutic intervention of aberrant PI3K/Akt activation in cancers, including neuroblastoma, is currently an area of high interest. So far, PI103 inhibits Akt phosphorylation, resulting in downregulation of Mcl-1 and cFLIP and decreased phosphorylation of Bim. PI103 and TRAIL cooperate to trigger cleavage of Bid into tBid and its insertion into mitochondrial membranes, Bax/Bak activation, mitochondrial outer membrane permeabilization (MOMP), caspase-3 activation, and apoptosis. A caspase-3–driven feedback amplification loop promotes caspase-8 and Bid cleavage.

Second, our data provide novel insights into the signal transduction pathways, which are regulated by PI3K inhibitors and which can be exploited to prime cancer cells for TRAIL-induced apoptosis. Thus, beyond neuroblastoma these results will likely have an important impact on the development of combination therapies with PI3K inhibitors also in other types of cancer.

Figure 6. PI103 cooperates with TRAIL to suppress neuroblastoma growth in vivo. A, SH-EP cells were seeded on the CAM of chicken embryos, allowed for 48 hours to form tumors and treated with 1 μmol/L PI103 and/or 10 ng/mL TRAIL for 2 days. Tumor growth was analyzed by hematoxylin and eosin–stained paraffin sections of the CAM. Shown are tumor area (left) and representative pictures of hematoxylin and eosin–stained sections of the CAM (right; bar, 1,000 μmol/L). Error bars, mean ± SD of 6 samples per group; *, P < 0.05. Similar results were obtained in 2 independent experiments. B, schematic overview of the mechanisms responsible for the cooperative induction of apoptosis by PI103 and TRAIL. PI103 inhibits Akt phosphorylation, resulting in downregulation of Mcl-1 and cFLIP and decreased phosphorylation of Bim. PI103 and TRAIL cooperate to trigger cleavage of Bid into tBid and its insertion into mitochondrial membranes, Bax/Bak activation, mitochondrial outer membrane permeabilization (MOMP), caspase-3 activation, and apoptosis. A caspase-3–driven feedback amplification loop promotes caspase-8 and Bid cleavage. ↑, upregulation/activation; ↓, downregulation; êê, inhibition. See text for more details.
In conclusion, this preclinical evaluation of a rational combination of 2 novel classes of targeted drugs, that is, the PI3K inhibitor PI103 and TRAIL, in relevant preclinical in vitro and in vivo models of neuroblastoma provides the molecular basis for the design of new combination therapies for the treatment of neuroblastoma. This strategy may help to overcome apoptosis resistance of neuroblastoma and other cancers with aberrant activation of PI3K/Akt.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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