The flavonoid kaempferol sensitizes human glioma cells to TRAIL-mediated apoptosis by proteasomal degradation of survivin

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Abstract
Resistance to tumor necrosis factor–related apoptosis-inducing ligand (TRAIL/Apo2L) limits its potential as a drug for cancer therapy. Here, we report that kaempferol, a bioactive plant flavonoid, sensitizes U251 and U87 glioma cells to TRAIL-mediated apoptosis. In contrast, U373 cells are not affected by kaempferol treatment. Treatment of kaempferol alone for 24 h did not induce apoptosis in the cell lines. We provide evidence that TRAIL-induced apoptosis is partially driven by kaempferol-mediated reduction of survivin protein levels. On kaempferol treatment, proteasomal degradation of survivin was observed. Inhibition of proteasomal degradation with MG132 in kaempferol-treated cells restored survivin protein levels in both glial cell lines. Consequently, overexpression of survivin attenuated TRAIL-kaempferol–induced apoptosis. In addition, we show that kaempferol mediates down-regulation of phosphorylated Akt, thereby further reducing survivin protein level. Furthermore, the blockage of the serine/threonine kinase Akt activity by kaempferol is important for inhibition of survivin because active phosphorylated Akt enhances the stability of survivin. However, we also show that the combined treatment of TRAIL and kaempferol induces cleavage (activation) of caspase-8, thereby exerting a proapoptotic effect independent of survivin known not to inhibit caspase-8 activation. Other effects induced by kaempferol were suppression of X-linked inhibitor of apoptosis proteins as the antiapoptotic members of the Bcl-2 family, Bcl-2, Bcl-xL, and Mcl-1 in a concentration-dependent manner. In summary, we showed that suppression of survivin is an essential mechanism in TRAIL-kaempferol–mediated apoptosis.

Introduction
Resistance to tumor necrosis factor–related apoptosis-inducing ligand (TRAIL/Apo2L) limits its potential as a drug for cancer therapy. TRAIL binds to the death receptors DR4/DR5, which subsequently interact with the adaptor protein FADD and procaspase-8, forming the death-inducing signaling complex. Procaspase-8 activation in the death-inducing signaling complex leads to cleavage of procaspase-3 and engagement of the cellular machinery associated with the type I extrinsic apoptotic pathway (1, 2). Activation of the intrinsic, mitochondrial-associated type II apoptotic pathway is another hallmark of TRAIL-induced cell death because TRAIL, through caspase-8, activates Bid and synergizes with agents that induce apoptosis exclusively through a type II mechanism (3). TRAIL has been shown to induce apoptosis in tumor cells but not in nonneoplastic cells (4). Several studies have shown that intracranial delivery of native human TRAIL suppresses the growth of human glioma xenografts in mice without host toxicity (5). Induction of apoptosis by TRAIL might be a promising therapeutic approach in cancer therapy. However, because not all tumor cells are sensitive to TRAIL, there is a need for the development of strategies to overcome TRAIL resistance. There are several means by which the connection between TRAIL receptor activation and the apoptotic machinery could be altered in TRAIL-resistant tumors.

The inhibitor of apoptosis proteins (IAP) are of particular interest because in the majority of human gliomas these proteins are overexpressed compared with nonneoplastic brain (6). The presence of high levels of antiapoptotic factors in cancer cells, but not normal cells, such as the IAPs may confer this resistance. Members of the IAP proteins [e.g., the chromosome X-linked IAP (XIAP)] contain one or more conserved regions termed baculoviral IAP repeat NH2-terminal domains and a COOH-terminal RING domain (7, 8). The baculoviral IAP repeat domains block caspase-3 and caspase-9, whereas the RING domain acts as an ubiquitin ligase to facilitate proteasomal degradation of caspases (9). With only one baculoviral IAP repeat domain and lacking a RING domain, survivin is the smallest member of the IAPs (10). Survivin expression is not or only barely detectable in most adult tissues. However, it is overexpressed in human tumors and high levels of survivin.
expression correlate with poor clinical outcome and resistance to radiation and chemotherapy (11, 12). Recently, it has been reported that survivin mediates inhibition of the mitochondrial-mediated and death receptor (TRAIL)-mediated apoptosis (13). Therefore, it is pivotal to find new substances that have the ability to suppress survivin and XIAP.

Epidemiologic studies in humans have shown that regular consumption of fruits and vegetables is associated with reduced risk of cancer (14–16). One possible reason for this is that many fruits and vegetables contain flavonoids, which have been reported to exert potential anticarcinogenic activities (16). Kaempferol is one of the most common flavonoids in nature. To date, numerous biological activities of flavonoids have been identified (17). Kaempferol is known to possess cancer-preventive properties (18–21) and to inhibit migration and invasiveness of glioma cells (22).

In this study, we investigated the effects of TRAIL in combination with kaempferol on human glioma cells. We hypothesized that treatment with kaempferol enhances TRAIL-induced apoptosis by down-regulation of XIAP and survivin.

Materials and Methods

Cell Culture, Reagents, Plasmids, and Transfections

Human glioblastoma cell lines U87, U251, and U373 were cultured in DMEM Glutamax-i4.5 g/L glucose (Invitrogen) with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen) and incubated at 37°C in a humidified atmosphere containing 10% carbon dioxide. Kaempferol, MG132, and LY294002 were obtained from Sigma. Cells lines were treated with the indicated amounts of kaempferol for 24 h or shorter periods. Recombinant human TRAIL/Apo2L was purchased from PeproTech. Transient transfection of U87 cells was achieved by Eugene transfection reagent (Roche Deutschland Holding GmbH) or by electroporation using Nucleofector I program U29 (Amaza AG). With electroporation, up to 80% transfection efficiency was achieved. The survivin wild-type plasmid pcDNA3-survivin was a gift from Dario Altieri (Department of Cancer Biology, University of Massachusetts Medical School, Worcester, MA) and has been described previously (23). Empty pcDNA3 was used as a negative control in our experiments. The plasmid pUSE-amp–active Akt (Millipore GmbH) containing myc-tagged active Akt and the empty control (pUSE-amp) were transfected into U87MG. The human short-term glioblastoma cultures used were established as described (24, 25). In brief, glioblastoma specimens WHO IV were obtained from the Department of Neurosurgery, University Hospital Heidelberg. Primary cultures were established by dissecting tissues in small pieces and transferring to plastic tissue culture flasks (Falcon, Becton Dickinson). Cells were cultured in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin at 37°C, with 5% CO2. Cells were grown to confluence with medium changes biweekly and were harvested by a brief incubation with trypsin/EDTA solution (Viralex, PAA). Mycoplasma contamination of the cell cultures was excluded by 4',6-diamidino-2-phenylindole staining (Roche Diagnostics). Glial origin of cultured cells was confirmed by binding of α-glial fibrillary acidic protein antibody (Dako). Contamination with endothelial cells was excluded by showing lack of binding of antibodies directed against CD31 (PharMingen) and against VIII (Dako). Contamination with neuronal cells was excluded by showing lack of binding of antibodies directed against neurofilament protein 70, 160, and 200 kDa (all from Progen).

Cell Viability

Cells were seeded into 96-well plates at a density of 2 × 104 per well in 100 μL tissue culture medium in triplicate. After 24-h incubation, allowing cells to adhere, cells were treated for 48 h either with kaempferol and TRAIL separately or in different combinations, as described in individual experiments. Cell viability was determined by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay.

MTS assay was done using CellTiter 96 AQueous Assay System from Promega. In this assay, the quantity of formazan product formed is directly proportional to the number of viable cells in the cultures. After 4 h in culture, the cell viability was determined by measuring the absorbance at 490 nm using a 550 Bio-Rad plate reader (Bio-Rad). The relative percentage of survival was calculated by dividing the absorbance of treated cells by that of the control in each experiment.

Small Interfering RNA Transfection

Survivin-specific small interfering RNAs (siRNA) were synthesized by Dharmacon, Inc. All siRNAs were duplexed, desalted, 2' deprotected, and purified (>80%) by Dharmacon. Cells (5 × 105) were seeded per well in a 12-well chamber and incubated at standard conditions for 24 h. Subconfluent cells were transfected with TransIT-TKO Transfection Reagent or siRNA at a final concentration of 60 nmol/L diluted in TransIT-TKO Transfection Reagent. Forty-eight hours after transfection, cells were treated with medium or 250 ng/mL TRAIL, and protein expression was evaluated 72 h after transfection by immunoblotting.

Western Blot

Twenty micrograms of protein diluted in NuPAGE sample buffer and reducing reagent (Invitrogen) were denatured at 95°C for 5 min and electrophoretically separated on ready-to-use 4% to 12% SDS-PAGE (Invitrogen). Proteins were blotted onto nitrocellulose membranes at 1.5 mA/cm² for 1.5 h (Invitrogen). After blocking in 0.5 mol/L Tris base (pH 7.4), 5% milk powder, 1.5 mol/L NaCl, and 0.05% Tween, the membranes were incubated with rabbit anti-human XIAP antibody (1:1,000 dilution; R&D Systems), anti-human cIAP-2 (Cell Signaling Technology, Inc.), rabbit anti-human survivin antibody (1:2,000 dilution; Cell Signaling Technology), rabbit anti-human Bcl-xL (Cell Signaling Technology), rabbit anti-human cleaved poly(ADP-ribose) polymerase (PARP; Cell Signaling Technology), rabbit anti-human procaspase-9 (Cell Signaling Technology), rabbit anti-human cleaved caspase-7 (Cell

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Signaling Technology), rabbit anti-human procaspase-8 (Cell Signaling Technology), rabbit anti-human Bid (Cell Signaling Technology), rabbit anti-human Bcl-2 (Cell Signaling Technology), or rabbit anti-human Mcl-1 (Cell Signaling Technology) overnight at 4°C. Staining with secondary horseradish peroxidase–conjugated anti-rabbit or anti-mouse antibodies at dilutions of 1:10,000 or 1:1,400, respectively (Amersham Biosciences), was followed by immunodetection with Western Blotting Detection System ECL (Amersham Biosciences). Protein signals were analyzed semiquantitatively using a computer-assisted image analysis system and the NIH gel analysis software. The sum of gray values of all pixels and the sum of densities were determined for each individual band. To normalize the means of sum of density values from different experiments, each value was given as the percentage of the control value. Data are reported as mean ± SD of n experiments. Significant differences were assessed by paired Student’s t test and P < 0.05 was considered significant.

**Real-time Reverse Transcription-PCR**

Total RNA was isolated using the RNA Nucleospin RNA II kit (Macherey-Nagel) according to the manufacturer’s instructions. The total RNA concentration was assessed using a NanoDrop photometer (Peqlab Biotechnologie). For cDNA synthesis, 1 μg of total RNA was used for reverse transcription (random primer) using RevertAid H Minus MuLV Polymerase (1 unit/μL), according to the manufacturer’s instructions (MBIFermentas). The primer sequences for survivin and glyceraldehyde-3-phosphate dehydrogenase were: survivin, 5'-CCCGATGACAACCCGATA-3' (forward) and 5'-CATCTGCTTCTTGACAGTGAG-3' (reverse); glyceraldehyde-3-phosphate dehydrogenase, 5'-TGCCTCTTTAGTTGTCATGCAG-3' (forward) and 5'-CCCGTTCAGCTCAGGGATGA-3' (reverse). All real-time PCRs were done in a 25 μL mixture containing 1:20 volume of cDNA preparation (1 μL), 1 × SYBR Green buffer (PE Applied Biosystems), 4 mmol/L MgCl₂, 0.2 μmol/L of each primer (BA67 and BA68), 0.2 mmol/L deoxynucleotide triphosphate mix, and 0.025 unit of AmpliTaq Gold thermostable DNA polymerase (Applied Biosystems). The PCR was run on an ABI PRISM Detection Instrument 7400. After 40 cycles, data reduction was done with Sequence Detection System Software (Applied Biosystems).

### Figure 1.

MTS cell viability assay after treatment with kaempferol, TRAIL, or the combination of both after 24 h of treatment. **A,** cell viability in U251, U87, and U373 cells after kaempferol treatment only. **B** to **D,** effect of kaempferol on TRAIL-induced cytotoxicity in U251, U87, and U373 human glioma cells. **E,** viability in NCH82, NCH89, NCH149, NCH37, and NCH210 human short-term glioblastoma cultures after kaempferol treatment only. **F,** effect of kaempferol on TRAIL-induced cytotoxicity in NCH82, NCH89, NCH149, NCH37, and NCH210 human short-term glioblastoma cultures. Cont., not treated; KP50, 50 μmol/L kaempferol; KP100, 100 μmol/L kaempferol; KP200, 200 μmol/L kaempferol; TR50, 50 ng/mL TRAIL; TR100, 100 ng/mL TRAIL; TR250, 250 ng/mL TRAIL. Asterisks, values that are different from the respective control. *, P < 0.05, t test; **, P < 0.01, t test.
Statistics
The data were expressed as mean ± SE of separate experiments (n ≥ 3) and compared by the two-tailed paired Student’s t test. Differences between two treatments were considered significant at P < 0.05.

Results
Kaempferol Sensitized U87 and U251, but not U373, Cells for TRAIL-Induced Apoptosis
First, we measured the effects of kaempferol, with or without addition of TRAIL, on cell viability by MTS assay. Kaempferol (200 μmol/L) alone did not inhibit cell viability in U87, U251, and U373 glioma cells in a dose-dependent manner within 24 h (Fig. 1A). Addition of 50, 100, and 250 ng/mL of TRAIL did not have a significant effect on cell viability (94 ± 3%, 95 ± 6%, and 91 ± 7%; Fig. 1B–D). Kaempferol enhanced the inhibitory effects of TRAIL on cell viability in U251 and U87 cells within 24 h (Fig. 1B–D). Compared with control cells, addition of 200 μmol/L kaempferol in combination with 50, 100, and 250 ng/mL of TRAIL decreased viability by 40 ± 2% (P < 0.01), 45 ± 4% (P < 0.01), and 68 ± 4% (P < 0.01) in U251 cells and by 46 ± 5% (P < 0.01), 52 ± 5% (P < 0.01), and 77 ± 7% (P < 0.01) in U87 cells, respectively. The viability of U373 cells did not show any significant change after treatment with TRAIL and kaempferol (Fig. 1A–D).

Kaempferol Sensitizes Human Short-term Glioblastoma Cultures for TRAIL-Induced Cytotoxicity
The cytotoxic effect of TRAIL in gliomas has been thoroughly established in tumor cell lines. So, we also investigated the therapeutic potential of TRAIL in human short-term glioblastoma cultures. Two of five human short-term glioblastoma cultures (NCH89 and NCH82) were sensitive to TRAIL treatment within 24 h. Addition of 100 ng/mL TRAIL to NCH89 and NCH82 decreased cell viability by 20 ± 4% and 80 ± 6%, respectively (Fig. 1F). NCH49, NCH210, and NCH37 were resistant to TRAIL treatment even with concentrations of up to 1,000 ng/mL (data not shown). Treatment of human short-term glioblastoma cultures with kaempferol did not alter cellular viability significantly (Fig. 1F). Two of five human short-term glioblastoma cultures (NCH89 and NCH149) were further sensitized to TRAIL-mediated cytotoxicity by kaempferol. Addition of 100 ng/mL TRAIL in combination with 200 μmol/L kaempferol to NCH89 and NCH149 yielded a decrease in viability by 52 ± 6% and 21 ± 3%, respectively (Fig. 1F).

Kaempferol Augments TRAIL-Induced Apoptosis through Caspase Activation
We used Western blotting to analyze the extrinsic and intrinsic apoptotic pathways in TRAIL-induced and TRAIL-kaempferol–induced apoptosis. We analyzed PARP, caspase-8, cleaved caspase-7, and Bid in U87MG, U251, and U373 after treatment with kaempferol, TRAIL, and the combination of both. Treatment of U87MG and U251 cells with kaempferol alone did not or only weakly induce cleavage of PARP, caspase-7, caspase-8, caspase-9, or Bid (Fig. 2A and B). However, exposure of cells to TRAIL (100 ng/mL) for 8 h resulted in an appearance of cleavage products of caspase-8, caspase-9, caspase-7, and PARP in U87 and U251 cells. Combining TRAIL with kaempferol increased significantly the level of cleavage products caspase-8, caspase-9, caspase-7, and PARP in U87 and U251 cells (Fig. 2A and B). As kaempferol enhanced the extrinsic apoptotic pathway as outlined by increased caspase activation, combination of both increased the cleavage of PARP, caspase-8, caspase-7, and caspase-9 (Fig. 2A and B).

Figure 2. Immunoblot showing the effect of kaempferol on TRAIL-induced proteolytic cleavage of PARP, caspase-7, caspase-8, caspase-9, and Bid in U251, U87, and U373 cells. U251 (A), U87 (B), and U373 (C) cells were treated for 8 h. Values on top of bands represent relative densities normalized to β-actin.
cleavage (activation) of caspase-8, we checked whether kaempferol could also enhance the activation of the intrinsic, mitochondrial-associated type II apoptotic pathway by TRAIL-mediated apoptosis. For that reason, we analyzed the expression of Bid, which is a substrate of active caspase-8 and synergizes with agents that induce apoptosis exclusively through a type II mechanism (Fig. 2A and B). Exposure of TRAIL (100 ng/mL) for 8 h to U87 and U251 cells decreased the full-length Bid (22 kDa) and led to appearance of cleaved caspase-9 (37 kDa; Fig. 2A and B). Furthermore, this decrease was enhanced through the combination of TRAIL and kaempferol. The level of PARP, Bid, caspase-7, and caspase-8 did not change significantly in U373 cells after treatment with TRAIL, kaempferol, or combination of both agents (Fig. 2C). Taken together, kaempferol enhances TRAIL-mediated apoptosis through the extrinsic and intrinsic apoptotic pathways.

Inhibition of Survivin and XIAP by Kaempferol

Because kaempferol augments TRAIL-induced apoptosis by activation of caspases, we sought to examine the effects of kaempferol on the expression of IAPs (XIAP and survivin) in U87, U251, and U373 cells (Fig. 3A–C). Cells were treated with kaempferol for 24 h, and the expression of IAPs was measured by Western blot analysis. Compared with control cells, 200 μmol/L kaempferol inhibited the expression of survivin and XIAP in U251 and U87 significantly (Fig. 2A and B). Survivin and XIAP were not suppressed in U373 cells (Fig. 3C). These data suggest that inhibition/cleavage of IAPs by kaempferol may be one of the mechanisms regulating apoptosis.

Regulation of Bcl-2 Family Members by Kaempferol

Bcl-2 family members regulate apoptosis induced by stress stimuli primarily at the level of mitochondria (27). We therefore examined the effects of kaempferol on the expression of Bcl-2 family members. Western blot analysis showed that on treatment with kaempferol the expression of the antiapoptotic proteins Bcl-2 and Mcl-1 was decreased significantly in U251 and U87 cells (Fig. 3). Bcl-xL was not influenced by kaempferol treatment in U251 and U87 cells (Fig. 3). Interestingly, expression of Bcl-xL and Mcl-1 was enhanced, whereas Bcl-2 was suppressed in U373 after treatment with kaempferol for 24 h (Fig. 3C).

Survivin-Specific siRNA Sensitizes U251 for TRAIL-Induced and TRAIL-Kaempferol–Induced Cytotoxicity

To further investigate the role of survivin in TRAIL-kaempferol–mediated apoptosis, we transfected U251 glioma cells with double-stranded siRNA oligonucleotides. Cells were harvested at 48 h after transfection, and survivin expression was analyzed by Western blot (Fig. 4A). Whereas survivin-specific siRNA dramatically suppressed survivin synthesis, control siRNA had no significant effect on survivin expression. Glioma cells transfected with nontargeted siRNA or without siRNA (mock transfection) revealed no effect on survivin expression (Fig. 4A). Next, we assessed the effect of survivin-specific siRNA on cell viability (Fig. 4C). Combining survivin-specific siRNA with kaempferol did not alter cell viability significantly compared with kaempferol-treated cells or controls (91 ± 6%). Survivin-specific siRNA sensitized U251 cells to TRAIL-induced cytotoxicity almost to the same extent as TRAIL-kaempferol–treated cells that were transfected with nontargeted siRNA (Fig. 4C). Addition of 50 ng/mL TRAIL for 24 h to survivin-specific siRNA-transfected U251 cells and addition of 50 ng/mL TRAIL in tandem with 200 μmol/L kaempferol to U251 cells that were transfected with nontargeted siRNA yielded a decrease of cell viability by 32 ± 5% (P < 0.01) and 36 ± 5% (P < 0.01), respectively (Fig. 4C). Interestingly, TRAIL-kaempferol–mediated cytotoxicity was also enhanced by survivin-specific siRNA compared with the nontargeted siRNA-transfected counterparts (Fig. 4B). Nontargeted siRNA-transfected and survivin-specific siRNA-transfected U251 cells that were treated with 50 ng/mL TRAIL and 200 μmol/L kaempferol yielded a decrease of cell viability by 35 ± 4% (P < 0.01) and 55 ± 5% (P < 0.01), respectively. Notably, the cell viability of survivin-specific siRNA-transfected U251 cells that were
treated with 50 ng/mL TRAIL and 200 μmol/L kaempferol was also significantly reduced compared with nontargeted siRNA-transfected U251 cells that received only treatment with 50 ng/mL TRAIL and 200 μmol/L kaempferol. In addition, we also down-regulated XIAP and found out that the effect was not as strong as for survivin (data not shown).

In summary, survivin is involved in kaempferol-mediated regulation of apoptosis. To prove that those effects above are specific for apoptosis, we used immunoblotting. Compared with U251 cells transfected with nontargeted siRNA, the cleavage products of the effector caspase-7 are significantly increased in survivin-specific siRNA-transfected U251 cells that were either untreated or treated with kaempferol, TRAIL, or the combination of both (Fig. 4B).

These data suggest that survivin is involved in transducing effects of kaempferol and TRAIL.

Overexpression of Survivin Attenuates TRAIL-Kaempferol–Mediated Cytotoxicity

Based on the above results, we investigated whether down-regulation of survivin actually mediates kaempferol-facilitated TRAIL-induced apoptosis. For that reason, we transiently transfected U251 cells with a plasmid containing wild-type survivin and an empty plasmid that served as a control. Transfection was confirmed by Western blotting (Fig. 5A). Overexpression in U251 cells significantly attenuated kaempferol-enhanced TRAIL-induced cytotoxicity (Fig. 5B). Addition of 100 ng/mL TRAIL in tandem with 200 μmol/L kaempferol to U251 cells that were transfected with the control plasmid yielded a decrease of cellular viability by 44 ± 6% (P < 0.01). The reduction of cellular viability was attenuated in U251 cells transfected with wild-type survivin with a decrease of cellular viability by only 10% in respect to the untreated control.

Inhibition of Survivin Expression by Kaempferol Is Mediated through Proteasomal Degradation and Akt

To investigate whether kaempferol regulates survivin expression on transcriptional level, we used reverse transcription-PCR. In both cell lines (U251 and U87), mRNA levels of survivin did not change significantly after treatment with kaempferol for 24 h (Fig. 6A).

One possible explanation would be that survivin would be degraded by the proteasome. To confirm this hypothesis, we treated the cells with the proteasome inhibitor MG132. U251 and U87 cells were treated with kaempferol (200 μmol/L), MG132 (5 μmol/L), or kaempferol and MG132 in combination. As expected, suppression of survivin in U251 and U87 cells was almost completely abolished by MG132 (Fig. 5C and D).

It is well known that elevated Akt activity protects cells from TRAIL-induced apoptosis (28, 29). We postulated that kaempferol inhibits Akt activity and consequently enhances TRAIL-induced cytotoxicity through suppression of survivin because it has been shown that Akt up-regulates survivin expression (30). The effect of kaempferol on Akt was compared with that of LY294002, a well-known specific inhibitor of phosphatidylinositol 3-kinase. U251 glioma cells were treated with LY294002 (50 μmol/L) and kaempferol (200 μmol/L) for 24 h (Fig. 6B). We observed that kaempferol induced dephosphorylation of Akt in U251 glioma cells. The dephosphorylation and suppression of Akt by LY294002 was accompanied by a reduction of survivin, similar to the effect of kaempferol (Fig. 6B). In addition, overexpression of phosphorylated Akt restored survivin levels in kaempferol-treated glioma cells. For that reason, we transiently transfected U251 glioma cells with a plasmid containing phosphorylated myc-tagged Akt and an empty control plasmid. Forty-eight hours later, the transfected cells were treated with 200 μmol/L kaempferol or left untreated. Two bands were detected in the myc-tagged active Akt-transfected cells: the endogenous phosphorylated Akt and the myc-tagged phosphorylated Akt. The lower band that represents endogenous
phosphorylated Akt is markedly suppressed by kaempferol (200 μmol/L) in both control vector and the myc-tagged active Akt-transfected cells (Fig. 6C). The data show that kaempferol-mediated inhibition of survivin not only enhances proteasomal degradation but also is mediated by Akt.

Discussion
Attempts to improve the survival of glioma patients also focus on targeting resistance to apoptosis. TRAIL almost selectively induces apoptosis in cancer cells with no or minimal toxicity on nonneoplastic cells (31). However, most cancer cells, including various glioma cells, are resistant to TRAIL therapy (32). We aimed at sensitizing glioma cells to TRAIL-mediated apoptosis. Among others, such attempts have been undertaken with Smac agonists, mammalian target of rapamycin inhibitors. The combination of TRAIL and Smac agonist proved to be successful in repressing glioma transplants in mice and the combination of TRAIL and rapamycin induced apoptosis in several glioma cell lines (29). We used the flavonoid kaempferol recently shown to have anticancer efficacy against a broad range of cancers in cell culture studies. Our findings suggest that kaempferol may be useful as an anticancer drug or as an adjunct in combination therapy (e.g., with TRAIL).

In this study, we showed for the first time that combined application of TRAIL and kaempferol strongly reduced viability of U251 and U87 glioma cells but failed to do so in U373 cells. In contrast, neither kaempferol nor TRAIL nor the combination of both induced apoptosis in nonneoplastic fibroblasts and Schwann cells (data not shown). Previous reports showed that U373 cells could not be sensitized by cycloheximide to TRAIL-mediated apoptosis (33, 34). The reason why U373 is highly TRAIL resistant is a matter of debate. It has been reported that U373 expresses only low levels of the initiator caspase-8, thereby leading to an insufficient activation of death-inducing signaling complex (35).

With regard to p53 status, it has been reported that cell lines retaining p53 function did not differ in their TRAIL sensitivity from p53-mutant cell lines. This is completely in line with our data as both U251 and U373 are p53 mutant and, independent of p53 status, only U251 could be efficiently sensitized through quercetin to TRAIL-mediated cytotoxicity. Kaempferol and TRAIL alone had no effect on cell viability (Fig. 1B–D). Furthermore, two of five human short-term glioblastoma cultures could be sensitized to TRAIL-mediated apoptosis by kaempferol (Fig. 1F). Although many tumor cell lines, including glioma cell lines, have been reported to be sensitive to TRAIL, it has been reported recently that freshly isolated tumor cells from glioma patients of all four WHO grades of malignancy were TRAIL resistant (32). Thus, TRAIL as a monotherapy agent might be falling short of expectations. Another disturbing discovery was that TRAIL treatment of TRAIL-resistant pancreatic cancer cells even increased tumor cell migration and metastatic spread in vivo and in vitro (36). So, those findings warrant that TRAIL should be used in combination with other substances (e.g., flavonoids). Unlike short-term treatment (24 h), long-term treatment (up to 96 h) of kaempferol leads to cytotoxicity in a variety of cancer cells (18, 19). The strong effect of simultaneous application of TRAIL and kaempferol shows a synergistic action of combined treatment. Kaempferol already has proven nontoxic in animal models (37). Thus, the drug kaempferol in tandem with TRAIL may be of interest on treating human glioma.

It has been reported that kaempferol modulates expression of genes involved in apoptosis (19, 20). Whether a cell dies in response to diverse apoptotic stimuli is determined largely by interactions between proteins of the Bcl-2 family. In our study, we detected a suppression of Bcl-2 and Mcl-1 in U251 and U87 cells. Bcl-xl was unaffected in U251 and mildly suppressed in U87 cells. Interestingly, the expression of Bcl-xl and Mcl-1 in U373 cells increased on kaempferol treatment, which could be one explanation why U373 cells were not sensitized to TRAIL-mediated apoptosis (35).

Figure 5. Overexpression of survivin in U251 cells attenuates kaempferol-facilitated TRAIL-mediated apoptosis and down-regulation of survivin is mediated by proteasomal degradation. A, Western blot showing survivin expression 48 h after transfection with pcDNA3-survivin. B, MTS cell viability assay in U251 cells 72 h after transfection and 24 h after treatment with 100 ng/mL TRAIL and 200 μmol/L kaempferol. C and D, Western blot shows inhibition of kaempferol-induced survivin expression by MG132 in U251 (G) and U87 (D) cells. MG0, 0 μmol/L; MG132, 5 μmol/L MG132. Values on top of bands represent relative densities normalized to β-actin. Asterisks, values that are different from the respective control. *, P < 0.05, t test; **, P < 0.01, t test.
apoptosis by kaempferol. Our results are in line with previous reports, as it has been shown by other groups that Bcl-2 was down-regulated on kaempferol treatment of A549 lung cancer cells (38).

IAPs block cell death both in vitro and in vivo by virtue of inhibition of distinct caspases. We found that both survivin and XIAP expression were strongly reduced in U251 and U87 cells on treatment with kaempferol and unaffected in U373. This may indicate a special sensitivity of U87 and U251 to kaempferol-mediated TRAIL activation because two potent members of the IAPs are down-regulated. Consequently, we showed that overexpression of survivin attenuated kaempferol-facilitated TRAIL-mediated apoptosis, thereby supporting the fact that suppression of survivin is a key mechanism through which kaempferol enhances TRAIL-mediated apoptosis. Thus, targeting of IAPs may be of special promise in glioma. We also identified two mechanisms through which survivin was regulated in glioma cells on treatment with kaempferol. Although the mRNA levels of survivin were not affected by kaempferol treatment, pretreatment of glioma cells with MG132 significantly inhibited kaempferol-induced down-regulation of survivin in U251 and U87 glioma cells, suggesting that kaempferol may promote proteasome-mediated degradation of survivin. Proteasome-mediated degradation of survivin as a mechanism of regulation has already been reported by other researchers (39). It was shown that survivin protein was unstable (half-life of ~ 30 min) and easily ubiquitinized followed by degradation through the proteasome (40). Akt, also referred to as protein kinase B or Rac, plays a critical role in controlling survival and apoptosis (41). Akt regulates apoptosis via the direct phosphorylation and inactivation of Bad, caspase-9, the forkhead transcription factors, and NF-κB. The Akt-survivin pathway has been well defined and implicated in the resistance of cancer cells to therapeutics and TRAIL (28,30). Kaempferol has been shown to inhibit Akt-1 and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase-mitogen-activated protein kinase pathway in A549 lung cancer cells (20). In line with previous reports, we showed in the present study that the inhibitory effect of kaempferol on survivin expression seems to result from suppression of Akt activity, as blockage of Akt by the chemical phosphatidylinositol 3-kinase inhibitor LY294002 and kaempferol similarly decreased survivin expression in U251 cells. In addition, we found that overexpression of phosphorylated active Akt restored survivin levels in kaempferol-treated glioma cells.

We were interested in the specific role of survivin in kaempferol- and TRAIL-mediated reduction of cell viability. To this end, we down-regulated survivin by siRNA treatment. Combined TRAIL and siRNA survivin treatment was almost as effective as combined TRAIL and kaempferol treatment. This effect was survivin siRNA specific because TRAIL and nontargeted siRNA had no effect on cell viability. Thus, in U251 and U87 cells, the kaempferol-mediated reduction of survivin expression may be of special functional importance. However, triple treatment of TRAIL, kaempferol, and siRNA survivin further reduced cell viability, indicating that the pleiotropic effect of kaempferol on apoptosis-related genes can act in synergy with survivin repression. Given the role of IAPs in the development and progression of solid tumors and hematologic malignancies, efforts are under way to develop therapeutic IAP inhibitors, with a focus on XIAP and survivin (42). Antisense oligonucleotides have been developed and are currently in phase I clinical trial (42). Our results suggest that the combination of kaempferol and TRAIL could be an effective approach for glioma therapy. It has been noticed recently that TRAIL could promote TRAIL-resistant cancer cell proliferation and metastasis (43). Thus, sensitization of TRAIL-induced cytotoxicity in cancer cells by a combination of TRAIL and kaempferol may be particularly relevant in retaining the cancer-killing activity and circumventing the cancer-promoting potential of TRAIL. In vivo experiments with animal models are needed to verify the efficacy of the apoptosis by kaempferol. Our results are in line with previous reports, as it has been shown by other groups that Bcl-2 was down-regulated on kaempferol treatment of A549 lung cancer cells (38).

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TRAIL and kaempferol combination for glioma therapy. In addition, our data indicate that survivin may be of special interest as a target in glioma.

Disclosure of Potential Conflicts of Interest
None of the authors has commercial considerations, such as an equity interest, patent rights, or corporate affiliations, including consultantships, for any product or process mentioned in the submission.

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References
The flavonoid kaempferol sensitizes human glioma cells to TRAIL-mediated apoptosis by proteasomal degradation of survivin


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