Legend to Supplemental Figures and Tables

Suppl. Fig. 1. Expression of Bcl-2 family proteins in glioblastoma cell lines.

(a) Constitutive expression of Bcl-2 family proteins was determined in glioblastoma cell lines by Western blotting. A representative experiment of three independent experiments is shown.

(b) BJ human fibroblasts were treated for 24 hours with 10 ng/ml TRAIL indicated concentrations of ABT-737. Cell viability was determined by MTT assay and is expressed as percentage of untreated controls. Data represent mean + SEM of three independent experiments performed in triplicate.

Suppl. Fig. 2. ABT-737 cooperates with TRAIL to suppress colony formation in a caspase-dependent manner.

T98G cells were treated for 12 hours with 2 ng/ml TRAIL and/or 5 μM ABT-737 in the presence or absence of 20 μM zVAD.fmk. Colonies were stained with crystal violet after 14 days and were counted under the microscope. One representative experiment and the percentage of colony numbers compared to untreated control are shown. Data represent mean + SEM of three independent experiments (**$P<0.001$).

Suppl. Fig. 3. No effect of ABT-737 on TRAIL-R1/2 expression, TRAIL-induced DISC formation or complex II assembly.

(a) U87MG (left panel) and U118MG (right panel) cells were treated for six hours with 5 ng/ml (U87MG) or 10 ng/ml (U118MG) TRAIL and/or 5 μM ABT-737. Surface expression of agonistic TRAIL receptors TRAIL-R1 and TRAIL-R2 was determined by fluorescence-conjugated antibodies and flow cytometry, isotype antibody was used as control. Mean + SEM of three independent experiments carried out in triplicate are shown.

(b) U87MG cells transduced with a vector containing IκBα-SR (black bars) or control vector (white bars) were treated for 24 hours with 5 ng/ml TRAIL and/or 5 μM ABT-737. Apoptosis

was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei. Mean + SEM of three independent experiments carried out in triplicate are shown.

(c) U87MG (left panel) and U118MG (right panel) cells were treated for 30 minutes with 1 μg/ml TRAIL, 1.25 μg/ml anti-Flag M2 and/or 5 μM ABT-737. TRAIL DISC was analyzed by immunoprecipitation (IP) as described in Materials and Methods, expression of TRAIL-R2, FADD and caspase-8 in lysates served as control. A representative experiment of three independent experiments is shown.

(d) U87MG was treated for four hours with 5 ng/ml TRAIL and/or 5 μM ABT-737. Complex II assembly was analyzed by immunoprecipitation (IP) of caspase-8 as described in Materials and Methods, expression of RIP1, FADD and caspase-8 in lysates served as control. A representative experiment of two independent experiments is shown.

Suppl. Fig. 4. Effect of ABT-737 and TRAIL on Mtch2 expression.

U87MG (upper panel) and U118MG (lower panel) cells were treated for indicated times with 5 ng/ml (U87MG) or 10 ng/ml (U118MG) TRAIL and/or 5 μM ABT-737. Expression of Mtch2 was analyzed by Western Blot. β-actin served as loading control. A representative experiment of two independent experiments is shown.

Suppl. Fig. 5. ABT-737 and TRAIL cooperate to promote mitochondrial insertion of tBid and mitochondrial outer membrane permeabilization in several glioblastoma cell lines.

In the left panels, A172, T98G and U138MG glioblastoma cells were treated with 2 ng/ml (A172, T98G) or 20 ng/ml (U138MG) TRAIL and/or 5 μM ABT-737 for five hours and Bid expression was analyzed in the cytosolic and mitochondrial fraction as described in Materials and Methods. A representative experiment of three independent experiments is shown. In the right panels, mitochondrial membrane potential was assessed by FACS analysis after

treatment with ABT-737 and/or TRAIL for indicated times. The percentage of cells with loss of mitochondria membrane potential with mean + SEM of three independent experiments carried out in triplicate are shown (*P<0.005).

Suppl. Fig. 6. Effect of Bim expression on ABT-737- and TRAIL-induced apoptosis.

(a) U118MG a cells were transduced with control vector (shCtrl) or a vector containing two different shRNA sequences against Bim (shBim_1 and shBim_2). Cells were treated for 24 hours with 10 ng/ml TRAIL and/or 5 μM ABT-737. Cell viability was determined by MTT assay and is expressed as percentage of untreated controls. Data represent mean + SEM of three independent experiments performed in triplicate.

(b) T98G cells were transduced with control vector (shCtrl) or a vector containing two different shRNA sequences against Bim (shBim_1 and shBim_2) or against Bid (shBid_2). Cells were treated for 24 hours with 2 ng/ml TRAIL and/or 5 μM ABT-737. Cell viability was determined by MTT assay and is expressed as percentage of untreated controls. Data represent mean + SEM of three independent experiments performed in triplicate.

Suppl. Fig. 7. Caspase-3 amplification loop contributes to ABT-737- and TRAIL-induced apoptosis.

U87MG (left panel) and U118MG (right panel) cells were transduced with control vector (shCtrl) or a vector containing two different shRNA sequences against caspase-3 (shC3_1 and shC3_2). Cells were treated for 24 hours with 5 ng/ml (U87MG) or 10 ng/ml (U118MG) TRAIL and/or 5 μM ABT-737. Cell viability was determined by MTT assay. Data represent mean + SEM of three independent experiments performed in triplicate (**P<0.001).

Suppl. Fig. 8. Schematic model of the synergistic action of ABT-737 and TRAIL.

ABT-737 and TRAIL act in concert to cleave Bid into tBid. TRAIL initiates cleavage of Bid

into tBid by activating caspase-8, while ABT-737 facilitates the accumulation of tBid at mitochondrial membranes by neutralizing Bcl-2 and Bcl-X_L. This enhances the crosstalk between the extrinsic and the intrinsic apoptosis pathways culminating in a mitochondrial amplification loop that leads to increased Bax activation, loss of mitochondrial membrane potential, the release of cytochrome c and Smac from mitochondria and caspase-dependent apoptosis. Also, the combination treatment of ABT-737 and TRAIL contributes to Bid cleavage in a feedback loop via increased caspase-3 activity. See text for more details.

Suppl. Tab. 1. *p53* and *PTEN* status of glioblastoma cell lines.

The *p53* and *PTEN* status of the glioblastoma cell lines used in this study is indicated according to Ishii et al. (17).

Suppl. Tab. 2. ABT-737 and TRAIL act in a synergistic manner to reduce cell viability.

Cells were treated for 24 hours with indicated concentrations of ABT-737 and TRAIL and cell viability was determined by MTT assay in glioblastoma cell lines (a) and in primary cultured glioblastoma cells (b). Combination index (CI) was calculated as described by Chou (24) using CalcuSyn software. CI <0.9 indicates synergism, 0.9-1.1 additivity and >1.1 antagonism.

Supplemental Materials and Methods

TRAIL DISC Immunoprecipitation

Immunoprecipitation of the TRAIL DISC was performed by incubating cells with Flag-tagged TRAIL (Alexis) (1µg/ml) and/or 5 µM ABT-737 or left untreated. After lysis using a buffer containing 50 mM Tris-HCl, 1% (v/v) Triton-X 100, 150 mM NaCl, protease inhibitor cocktail (Roche), 1 µg/ml Flag-tagged TRAIL was also added to the ABT-737-treated and untreated samples. The TRAIL receptor-associated DISC was immunoprecipitated using 1.25 µg/ml mouse-anti Flag M2 antibody (Sigma). Elution of the precipitate was done adding 10 µl pan-mouse IgG Dynabeads (Invitrogen) and overnight rotation. Samples were washed three times with washing buffer I (50 mM Tris-HCl, 500 mM NaCl, 1% (v/v) Igepal CA-630 (NP40) (Sigma)), and once with washing buffer II (25 mM Tris-HCl) and analyzed by Western blot for expression of TRAIL-R2, FADD and caspase-8.

For immunoprecipitation of caspase-8, cells were lysed in NP40 buffer (10 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, supplemented with a protease inhibitor tablet (Roche). 1 mg of protein was incubated with 10 µg mouse anti-Caspase-8 antibody (Alexis, Grünberg, Germany) overnight at 4° C followed by the addition of 20 µl pan-mouse IgG Dynabeads (Invitrogen), then incubated for two hours at 4° C and washed with NP40 buffer and analyzed by Western blot for expression of RIP1, FADD and caspase-8.

Determination of TRAIL receptor expression.

Cells were incubated with mouse anti-human TRAIL-R1 or -R2 monoclonal antibodies (Alexis) for 30 minutes at 4° C, washed in PBS containing 1% FCS, incubated with rabbit anti-mouse F(ab′)2 IgG/biotin (BD Biosciences) for 20 minutes at 4° C in the dark, washed in PBS containing 1% FCS, incubated with streptavidin-phycoerythrin (BD Biosciences) for 20 minutes at 4° C in the dark and analyzed by flow cytometry.

Western blot analysis

The following antibodies were used for Western blot analysis: Noxa (Alexis); Bim (Cell Signaling); Bcl-2, Bak, Bcl-X_L, Smac, cytochrome c (BD Biosciences); Bid (R&D Systems Inc.); TRAIL-R2 (Millipore); Mcl-1 (Stressgen, Ann Arbor, MI); Puma, Bax6A7 (Sigma); MTCH2 (Abgent, San Diego, CA).