Supplemental Figure Legends

Suppl. Fig. 1. ABT-737 induces apoptosis in RMS cells.

A. TE671 cells were treated with 10 μM ABT-737 for six hours. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei. Representative histograms of three independent experiments are shown.

B. TE671 cells were treated with 10 μM ABT-737 for indicated times. Cell viability was determined by MTT assay and is expressed as percentage of untreated controls. Data represent mean + SD of three independent experiments performed in triplicate.

Suppl. Fig. 2. Morphological features of GX15-070- or ABT-737-induced cell death.

TE671 cells were treated with 200 nM GX15-070 for 48 hours or 10 μM ABT-737 for six hours. Morphological features of GX15-070- or ABT-737-induced cell death were assessed by phase contrast microscopy (A) or by Hoechst staining of nuclei and fluorescence microscopy (B). Representative pictures are shown.

Suppl. Fig. 3. ABT-737 induces caspase-dependent cell death.

TE671 and RMS13 cells were treated for 48 hours with 10 μM ABT-737 in the presence or absence of 20 μM zVAD.fmk. Cell viability was determined by MTT assay and is expressed as percentage of untreated controls (A). Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei (B). Data represent mean + SD of three independent experiments performed in triplicate.

Suppl. Fig. 4. Effect of Bcl-2 knockdown or overexpression on GX15-070-induced cell death.

A and B. TE671 and RMS13 cells were transfected with control siRNA (siCtrl) or two distinct siRNA sequences against Bcl-2 (siBcl-2#1, siBcl-2#2). Expression of Bcl-2 was determined by Western blotting (A). Cell viability was determined by MTT assay after treatment with indicated concentrations of GX15-070 for 72 hours and is expressed as percentage of untreated controls (A).

C and D. TE671 and RMS13 cells were transfected with pMSCV empty vector (EV) or pMSCV vector containing Bcl-2. Expression of Bcl-2 was determined by Western blotting (C). Cell viability was determined by MTT assay after treatment with indicated concentrations of GX15-070 for 72 hours and is expressed as percentage of untreated controls (D).

In (B, D) data represent mean + SD of three independent experiments performed in triplicate.

Suppl. Fig. 5. Scheme of GX15-070-induced cell death.

GX15-070 triggers the formation of autophagosomes and the interaction of Atg5, a component of autophagosomal membranes, with the necrosome, a complex containing FADD, RIP1 and RIP3, thereby forming a cytosolic cell death signaling platform that initiates necroptosis. Inhibition of autophagosome formation by knockdown of Atg5 or Atg7 abrogated GX15-070-induced cell death. Also, Atg5 is critically required for the assembly of the necrosome on autophagosomes, since silencing of Atg5 prevents the formation of the Atg5/FADD/RIP1/RIP3 complex. RIP1 and RIP3 are necessary for GX15-070-induced cell death downstream of autophagosome formation, since genetic or pharmacological inhibition of RIP1 or

RIP3 blocks GX15-070-induced cell death and suppression of longterm clonogenic growth without affecting autophagosome generation. See text for more details.

Supplemental Materials and Methods

Microscopy

Morphological alterations after treatment were analyzed by phase contrast microscopy using an inverted microscope (Olympus). For Hoechst staining, cells were stained after treatment with 1 µg/ml Hoechst dye at 37°C for 15 minutes and analyzed by a fluorescent microscope (Olympus).

Bcl-2 overexpression

For Bcl-2 overexpression, Phoenix cells were transfected with 20 µg of pMSCV empty vector or pMSCV vector containing murine Bcl-2 using calcium phosphate transfection. Virus containing supernatant was collected after 48 hours and filtered through a 45 µm filter. Cells were transduced by centrifugation at 1000xg for 1 hour at room temperature in the presence of 8 µg/ml polybrene and selected with 20 µg/ml Blasticidine.