Supplementary Figure Legends

Figure S1. FCS stimulation increases astrocytic and neuronal differentiation in NSCs

(A) GBM9 and GBM10 cells were analyzed for protein expression of PTEN and p53 by Western blotting; MDA-MB-231 cells were used as positive control for mutant p53 and wild-type PTEN expression. Expression of β-actin served as loading control.

(B, C) NSCs were treated for one week with 1 μM BV6, 2% FCS or DMSO. Cells were dissociated and stained with anti-GFAP antibody or isotype control for FACS analysis. A representative of three independent experiments (B) and the percentage of GFAP positive cells with mean + SEM of three independent experiments (C) are shown; *P<0.05

(D, E) NSCs were treated for one week with 1 μM BV6, 2% FCS or DMSO. Cells were dissociated and stained with anti-β−III-tubulin antibody or isotype control for FACS analysis. A representative of three independent experiments (D) and the percentage of β−III-tubulin positive cells with mean + SEM of three independent experiments (E) are shown; *P<0.05

Figure S2. Effect of Smac mimetic on cell proliferation

Cells were treated for indicated times with BV6 (GBM9: 0.6 μM, GBM10 and NSC: 1 μM) or DMSO. Cell number was quantified relatively to the number of phycoerythrine fluorescent beads by FACS analysis. Mean + SEM of three independent experiments performed in triplicate are shown; n.s.: not significant.

Figure S3. Smac mimetic activates NF-κB in NSCs

NSCs were treated with 1 μM or DMSO for seven days. Expression levels of p100 and p52 were analyzed by Western blotting. Expression of GAPDH served as loading control.

Figure S4. Effect of Smac mimetic on cell viability, cell death, cell proliferation and cell cycle of GBM10 cells overexpressing IκBα-SR

(A) GBM10 cells stably expressing IκBα-SR or vector control were treated for 48 hours with 1 μM BV6 or DMSO; stimulation with 10 ng/ml TNFα for one hour was used as positive control. Nuclear extracts were analyzed by EMSA for NF-κB DNA-binding activity. A representative of two independent experiments is shown.

(B) GBM10 cells stably expressing IκBα-SR or vector control were treated for one week with 1 μM BV6 or DMSO. Cell viability was measured by MTT assay and is expressed as fold increase of untreated controls.

(C) GBM10 cells stably expressing IκBα-SR or vector control were treated for one week with 1 μM BV6 or DMSO. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium-iodide-stained nuclei.

(D) GBM10 cells stably expressing IκBα-SR or vector control were treated for one week with 1 μM BV6 or DMSO. Cell cycle was analyzed by flow cytometry using propidium-iodide-stained nuclei. The percentage of cells in G1, S and G2 phases is shown.

(E) GBM10 cells stably expressing IκBα-SR or vector control were treated for one week with 1 μM BV6 or DMSO. Cell number was quantified relatively to the number of phycoerythrine fluorescent beads by FACS analysis.

In (B-E) mean + SEM of three independent experiments performed in triplicate are shown; n.s.: not significant.

Figure S5. Smac mimetic-induced astrocytic differentiation is blocked in GBM9 cells overexpressing IκBα-SR

(A) GBM9 cells were transduced with IκBα-SR or vector control and were analyzed for expression of IκBα by Western blotting. GAPDH expression served as a loading control.

(B) GBM9 cells stably expressing IκBα-SR or vector control were treated for ten days with 0.6 μM BV6 or DMSO. Cell viability was measured by MTT assay and is expressed as fold increase of untreated controls. Mean + SEM of two independent experiments performed in triplicate are shown.

(C) GBM9 cells stably expressing IκBα-SR or vector control were treated for ten days with 0.6 μM BV6 or DMSO. Cells were dissociated and stained with anti-GFAP antibody and isotype control for FACs analysis. The percentage of GFAP-positive cells is presented (C). Mean + SEM of two independent experiments is shown.

Figure S6. Smac mimetic induces differentiation of GBM CSLCs independently of TNFR1

(A) GBM10 cells were lentivirally transduced with shRNA against TNFR1 or vector control and knockdown of TNFR1 was assessed by Western blotting. β-actin expression served as loading control.

(B) GBM10 cells with TNFR1 knockdown or control cells were treated for one week with 1 μM BV6 or DMSO. Cell viability was measured by MTT assay and is expressed as fold increase of untreated controls.

(C) GBM10 cells with TNFR1 knockdown or control cells were treated for one week with 1 μM BV6 or DMSO. Cells were dissociated and stained with anti-GFAP antibody and isotype control for FACs analysis. The percentage of GFAP-positive cells is presented. Mean + SEM of three independent experiments is shown. ***P*<0.01; n.s.: not significant.

Figure S7. FCS-induced differentiation decreases CD133 expression in NSCs

(A, B) NSCs were treated for seven days with 1 μM BV6, 2% FCS or DMSO, dissociated and stained with anti-CD133-PE antibody or isotype control for FACS analysis. A representative of three independent experiments (A) and the percentage of CD133 positive cells are shown (B).

(C) After seven days of suspension culture, NSC spheres were dissociated and stained with anti-CD133-PE antibody or isotype control for FACS analysis. A representative of three independent experiments is shown.

(D) Quantification of CD133-positive NSCs after seven days of sphere culture and monolayer culture from FACS data shown in (A) and (C).

In (B, D) mean + SEM of three independent experiments is shown; **P*<0.05; n.s.: not significant.

Figure S8. Various IAP inhibitors activate NF-κB, stimulate cell elongation, enhance astrocytic differentiation and reduce stem cell markers of GBM CSLCs.

(A) GBM9 cells were treated for ten days with indicated concentrations of IAP inhibitors 2 and 3. Cell viability was measured by MTT assay and is expressed as fold increase of untreated controls.

(B) GBM9 cells were treated for ten days with 0.01 μM IAP inhibitors 2 and 3 or DMSO. Expression of cIAP1, cIAP2, XIAP and p52/p100 was analyzed by Western blotting. Expression of GAPDH served as loading control.

(C, D) GBM9 cells were treated for ten days 0.01 μM IAP inhibitors 2 and 3 or DMSO. Cell morphology was analyzed by phase-contrast microscope; scale bar: 100 μm (C). Cell elongation was quantified by measuring cell length and width and by calculating cell elongation index (length/width); fold increase in cell elongation index in the presence and absence of IAP inhibitors is shown (D).

(E) GBM9 cells were treated for ten days with 0.01 μM IAP inhibitors 2 and 3 or DMSO. Cells were then dissociated and stained with anti-GFAP, anti-β-III-tubulin, anti-CD133 antibodies or isotype control for FACS analysis. The percentage of GFAP, β-III-tubulin and CD133 positive cells is presented.

In (A, D, E), mean + SEM of three independent experiments are shown; *P<0.05; **P< 0.01; ***P<0.001; n.s.: not significant.

Supplementary Materials and Methods

IAP inhibitor 2 corresponds to compound 11 described by Oost et al. (1) and IAP inhibitor 3 was described by Chao et al. (2) and they were kindly provided by Pfizer (Groton, CT, USA). Anti-PTEN (Cell Signaling) and anti-p53 (BD Biosciences) antibodies were used for Western blot analysis.

References:

1. Oost TK, Sun C, Armstrong RC, Al-Assaad AS, Betz SF, Deckwerth TL et al. Discovery of potent antagonists of the antiapoptotic protein XIAP for the treatment of cancer. *J Med Chem.* 2004; **47**: 4417-4426

2. Chao B, Deckwerth TL, Furth P, S., Linton S, D., Spada A, P., Ullman B, R. et al., inventors; U.S. Patent PCT/US2005/024700. United States patent PCT/US2005/024700. 2006 16.02.