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Preclinical Development

Molecular Cancer Therapeutics

NF-κB Is Required for Smac Mimetic-Mediated Sensitization of Glioblastoma Cells for γ-Irradiation–Induced Apoptosis

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Abstract

Evasion of apoptosis contributes to radioresistance of glioblastoma, calling for novel strategies to overcome apoptosis resistance. In this study, we investigated the potential of the small molecule Smac mimetic BV6 to modulate radiosensitivity of glioblastoma cells. Here, we identify a novel proapoptotic function of NF-κB in γ-irradiation–induced apoptosis of glioblastoma cells by showing, for the first time, that NF-κB is critically required for Smac mimetic–mediated radiosensitization. BV6 significantly increases γ-irradiation–triggered apoptosis in several glioblastoma cell lines in a dose- and time-dependent manner. Calculation of combination index (CI) reveals that the interaction of BV6 and γ-irradiation is highly synergistic (CI < 0.3). Molecular studies show that BV6 stimulates NF-κB activation, which is critical for radiosensitization, because genetic inhibition of NF-κB by overexpression of the dominant-negative superrepressor IκBα-SR significantly decreases BV6- and γ-irradiation–induced apoptosis. Also, the BV6-mediated enhancement of γ-irradiation–triggered caspase activation, drop of mitochondrial membrane potential, and cytochrome *c* release is abolished in cells overexpressing IκBα-SR. Similarly, NF-κB inhibition by ectopic expression of a kinase dead mutant of IKKβ prevents the BV6-mediated sensitization for γ-irradiation. The clinical relevance is underscored by experiments with primary tumor samples showing that BV6 sensitizes primary cultured glioma cells as well as glioblastoma-initiating cancer stem cells derived from surgical specimens for γ-irradiation. In conclusion, we identify NF-κB as a critical mediator of Smac mimetic-conferred radiosensitization of glioblastoma cells. These results have important implications for the development of Smac mimetic–based combination protocols for radiosensitization of glioblastoma. *Mol Cancer Ther; 10(10); 1867–75. ©2011 AACR.*

Introduction

Glioblastoma is the most common primary brain tumor with a very poor prognosis (1). Resistance of glioblastoma to current treatment protocols, including radiotherapy, represents an ongoing challenge despite intensive treatment regimens (2). This highlights the need to develop novel approaches to increase the sensitivity of glioblastoma to current therapies to improve the dismal prognosis of this cancer (3).

Apoptosis is the intrinsic death program of the cell that controls normal tissue homeostasis (4). Apoptosis can be triggered via death receptors or mitochondria, usually resulting in the activation of caspases (5). The mitochondrial pathway of apoptosis is initiated by the release of cytochrome *c* and second mitochondria-derived activator of caspase (Smac)/direct inhibitor of apoptosis (IAP) binding protein with low pI (DIABLO) from mitochondria into the cytosol (6). Cytochrome *c* triggers caspase-3 activation via formation of the apoptosome complex, whereas Smac/DIABLO promotes apoptosis by neutralizing IAP proteins (6).

Evasion of apoptosis is one of the hallmarks of human cancers including glioblastoma (7) and also contributes to chemo- or radioresistance because therapy-induced cytotoxicity is mediated, to a large extent, by induction of cell death, including apoptosis in cancer cells (5). Apoptosis signaling may be disrupted by aberrant expression of antiapoptotic proteins (8), for example, by high levels of IAP proteins (9). IAP proteins all harbor a baculovirus IAP repeat (BIR) domain, whereas a RING finger domain with E3 ubiquitin ligase activity, which mediates (auto) ubiquitination and proteasomal degradation, is present in only some family members such as X-linked inhibitor of apoptosis (XIAP), cellular inhibitor of apoptosis 1 (cIAP1), and cIAP2 (9). XIAP binds to and inhibits caspase-3, -7, and -9 via its BIR3 domain and the linker region

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preceding BIR2 domain (9). cIAP1 and cIAP2 function as E3 ubiquitin ligases via their RING finger domain (9). Smac mimetics have been shown to trigger autoubiquitination and proteasomal degradation of cIAPs by stimulating their E3 ubiquitin ligase activity, thereby promoting NF-κB activation and TNFα-dependent cell death, besides neutralizing the inhibitory function of XIAP (10–12). Therefore, therapeutic targeting of IAP proteins by Smac mimetics may offer new possibilities to bypass resistance, for example, resistance to radiation-induced cell death.

The transcription factor NF-κB acts as a dimer consisting of proteins of the NF-κB/Rel family (13). In the canonical pathway, the subunits p65/p50 form the classical dimer that usually mediates transcriptional activation of target genes (13). Upon stimulation, the IκB kinase complex (IKK) becomes activated, which initiates the proteasomal degradation of IκBα, freeing NF-κB to translocate to the nucleus (13). Under many circumstances, NF-κB negatively regulates apoptosis via transcriptional activation of antiapoptotic proteins (13).

We previously reported that Smac peptides, which antagonize XIAP, sensitize glioblastoma cells for TRAIL-induced apoptosis *in vitro* and *in vivo* (14). In addition, we showed that genetic or pharmacologic inactivation of XIAP increases radiation-induced apoptosis in glioblastoma, neuroblastoma, and pancreatic carcinoma cells (15–17). To translate the concept of targeting IAP proteins for radiosensitization into a clinically applicable approach to improve the efficacy of radiotherapy in glioblastoma, we investigated in this study the therapeutic potential of BV6 for radiosensitization of glioblastoma.

Materials and Methods

Cell culture and reagents

Glioblastoma cell lines U87MG, A172, and T98G were obtained from the American Type Culture Collection that carries out cell line authentication by short tandem repeat analysis. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies Inc.) supplemented with 10% fetal calf serum (Biochrom), 1 mmol/L glutamine (Biochrom), 1% penicillin/streptavidin (Biochrom), and 25 mmol/L HEPES (Biochrom) as described (18). U87MG, A172, and T98G cell lines harbor PTEN mutation and are either p53 wildtype (U87MG, A172) or p53 mutant (T98G; ref. 19). Primary cultured glioblastoma cells and glioblastoma-initiating cancer stem cells obtained from fresh primary glioma specimens were cultured as described using serum-free medium containing 20 μg/mL epidermal growth factor and 10 μg/mL basic fibroblast growth factor for glioblastoma-initiating cancer stem cells (17, 18, 20). The study was approved by the Ethics Committee, Medical Faculty, University of Ulm. The bivalent Smac mimetic BV6 has previously been characterized and the structure of the compound has previously been published (Supplementary Fig. S1; ref. 11). BV6 was kindly provided by Genentech Inc. All chemicals were purchased from Sigma unless indicated otherwise. *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk) was obtained from Bachem, Enbrel from Pfizer.

Determination of apoptosis

Cells were treated with γ-irradiation (Nuclear Data, CS-137, 44Tbq, 2 Gy/min) at indicated doses and incubated for indicated times in the presence of BV6 or dimethyl sulfoxide (DMSO) as solvent. The radiation was delivered at the start of the experiment at the beginning of the BV6 exposure time. Apoptosis was determined by fluorescence-activated cell sorting analysis (FACScan; BD Biosciences) of DNA fragmentation of propidium iodide–stained nuclei as described (21).

Clonogenic survival assay

The clonogenic colony formation assay was done on single-cell suspension as described previously (22). Briefly, cells were plated in complete DMEM medium into 6-well plates (BD Biosciences) and 24 hours later, were treated with increasing concentrations of BV6 (0.1; 0.5; 1.0; 1.5 μmol/L), with DMSO or left untreated (mock). After an additional 4 hours, cells were irradiated at room temperature with single doses of X-ray (0, 2, 6, and 10 Gy) using a linear accelerator (SL 75/5; Elekta) with 6 megaelectron volt photons/100-cm focus-surface distance with a dose rate of 4.0 Gy/min. After 11 to 14 days, colonies were stained with methylene blue solution for 30 minutes and counted. Calculation of survival fractions (SF) was done using the equation SF = colonies counted/cells seeded × (PE/100), taking into consideration the individual plating efficiency (PE). Survival variables α and β were fitted according to the linear quadratic equation $SF = \exp[-\alpha\, x\, D - \beta\, x\, D^2]$ with D = dose using EXCEL software (Microsoft). All experiments were repeated at least 3 times. Radiation enhancement ratios at 50% and 10% survival were calculated by transforming the above mentioned equation using α and β values of the individual survival curves.

Determination of mitochondrial membrane potential and cytochrome *c* release

Tetramethylrhodamine methylester perchlorate (TMRE; 1 μmol/L; Sigma) was used to measure the mitochondrial transmembrane potential. Cells were incubated for 30 minutes at 37°C in the presence of the fluorochrome and immediately analyzed by flow cytometry. Cytochrome *c* release was determined in permeabilized cells using mouse anticytochrome *c* monoclonal antibody (BD Biosciences) as described (23).

Western blot analysis

Western blot analysis was done as described previously (18) using the following antibodies: mouse anti–caspase-8 (ApoTech Corporation), rabbit anti–caspase-3 (Cell Signaling), mouse anti-XIAP from BD

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Biosciences, goat anti–cIAP1 from R&D Systems, Inc., mouse anti–β-actin (Sigma), or mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; HyTest) followed by goat–antimouse or goat–antirabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology). Enhanced chemiluminescence was used for detection (Amersham Bioscience).

Retroviral transduction

Retroviral transduction of dominant-negative IκBα superrepressor (IκBα-SR) or kinase dead IKKβ (IKKβ−KD) was done as previously described (24) using the pCFG5-IEGZ retroviral vector system. In brief, PT67 producer cells (Clontech) were transfected with pCFG5-IEGZ vectors using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendation and selected with 0.25 mg/mL Zeocin (Invivogen). Stably transduced glioblastoma cell lines overexpressing IκBα-(S32; 36A) or IKKβ-KD were obtained by retroviral spin transduction and subsequent selection with Zeozin.

Nuclear protein extraction and electrophoretic mobility shift assay

Nuclear extracts were prepared as previously described (25). In brief, cells were washed, scraped, and collected by centrifugation at 1,000 × *g* for 5 minutes at 4°C. Cells were resuspended in low salt buffer, allowed to swell on ice for 12 minutes, followed by addition of a 10% Igepal CA-630 solution and centrifugation. The pelleted nuclei were resuspended in high salt buffer and nuclear supernatants were obtained by centrifugation. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce). For electrophoretic mobility shift assay (EMSA), the following oligomers were used: NF-κB 5′-AGTTGAGGGGACTTTCCCAGGC-3′ (sense), Sp1- 5′-ATTCGATCGGGGCGGGGCGAG-3′ (sense). Single-stranded oligonucleotides were labeled with γ-[^{32}P]-ATP by T4-polynucleotide kinase (MBI Fermentas GmbH), annealed and purified on Sephadex columns (Micro Bio-Spin P30; Biorad Laboratories). Binding reactions containing 5 μg nuclear extract, 1 μg Poly(dI:dC; Sigma), labeled oligonucleotide (10,000 cpm) and 5× binding buffer were incubated on ice and resolved by electrophoresis in nondenaturing 6% polyacrylamide gels and assessed by autoradiography. For supershift experiments, nuclear extracts were preincubated on ice with the following antibodies: p-50 (sc-7178X), p-65 (sc-372X), and cRel (sc-70X), all from Santa Cruz Biotechnology. Representative EMSAs are shown.

Statistical analysis

Statistical significance was assessed by Student's *t* test using Winstat software (R. Fitch Software). Drug interactions were analyzed by the combination index (CI) method based on that described by Chou (26) using CalcuSyn software (Biosoft). CI < 0.9 indicates synergism, <0.3 high synergism, 0.9 to 1.1 additivity, and >1.1 antagonism.

Results

Smac mimetic sensitizes glioblastoma cells for γ-irradiation–induced apoptosis

To explore the therapeutic potential of small molecule Smac mimetics for radiosensitization of glioblastoma, we selected BV6, a bivalent Smac mimetic that antagonizes XIAP, cIAP1, and cIAP2 (11). For combination experiments with γ-irradiation, we used a concentration of BV6 that triggered minimal apoptosis as single agent (Fig. 1A–C). Importantly, the addition of BV6 significantly enhanced γ-irradiation–induced apoptosis in a dose-dependent manner in several glioblastoma cell lines, as determined by the analysis of DNA fragmentation (Fig. 1A–C). Apoptotic cell death was confirmed by Annexin V staining (Supplementary Fig. S2A). Calculation of CI revealed that BV6 cooperated in a highly synergistic manner with γ-irradiation to trigger apoptosis (Fig. 1D, Supplementary Table S1). Furthermore, the cooperative induction of apoptosis by the combination treatment with BV6 and γ-irradiation occurred in a time-dependent fashion (Fig. 1E). To explore whether BV6 exerts an effect on long-term survival following radiation, we carried out colony assays. We selected 4 concentrations of BV6 for these experiments (0.1–1.5 μmol/L BV6) after determining the plating efficiency following treatment with BV6 (data not shown). BV6 potentiated the suppression of colony formation upon irradiation (Fig. 1F–H) and increased the radiation enhancement ratio (Table 1). This effect was most pronounced in the cell line A172 and gradually decreased in the cell lines T98G and U87MG, however, reaching also a level of significance following irradiation with higher doses in the latter 2 cell lines. This indicates that BV6 markedly increases radiation-induced apoptosis in all 3 glioblastoma cell lines, whereas the BV6-mediated sensitization for suppression of colony formation was most pronounced in A172 cells. Analysis of LC3 lipidation as a marker of autophagy revealed that treatment with BV6 and/or γ-irradiation had little effect on the induction of autophagy (Supplementary Fig. S2B). Together, this set of experiments shows that the Smac mimetic BV6 primes glioblastoma cells for γ-irradiation–induced apoptosis.

BV6 triggers caspase-dependent apoptosis

To gain insights into the underlying molecular mechanisms that mediate the synergistic action of BV6 and γ-irradiation, we tested the effect of distinct pharmacologic inhibitors that interfere with individual pathways. The addition of the broad-range caspase inhibitor zVAD.fmk significantly reduced BV6- and γ-irradiation–induced apoptosis (Fig. 2A), showing that caspase activity was required for apoptosis induction. Next, we tested the involvement of TNFα, which has been implicated in BV6-mediated cell death (10–12, 27). The addition of the TNFα antagonistic antibody Enbrel did not confer protection against BV6- and γ-irradiation–induced apoptosis, although it

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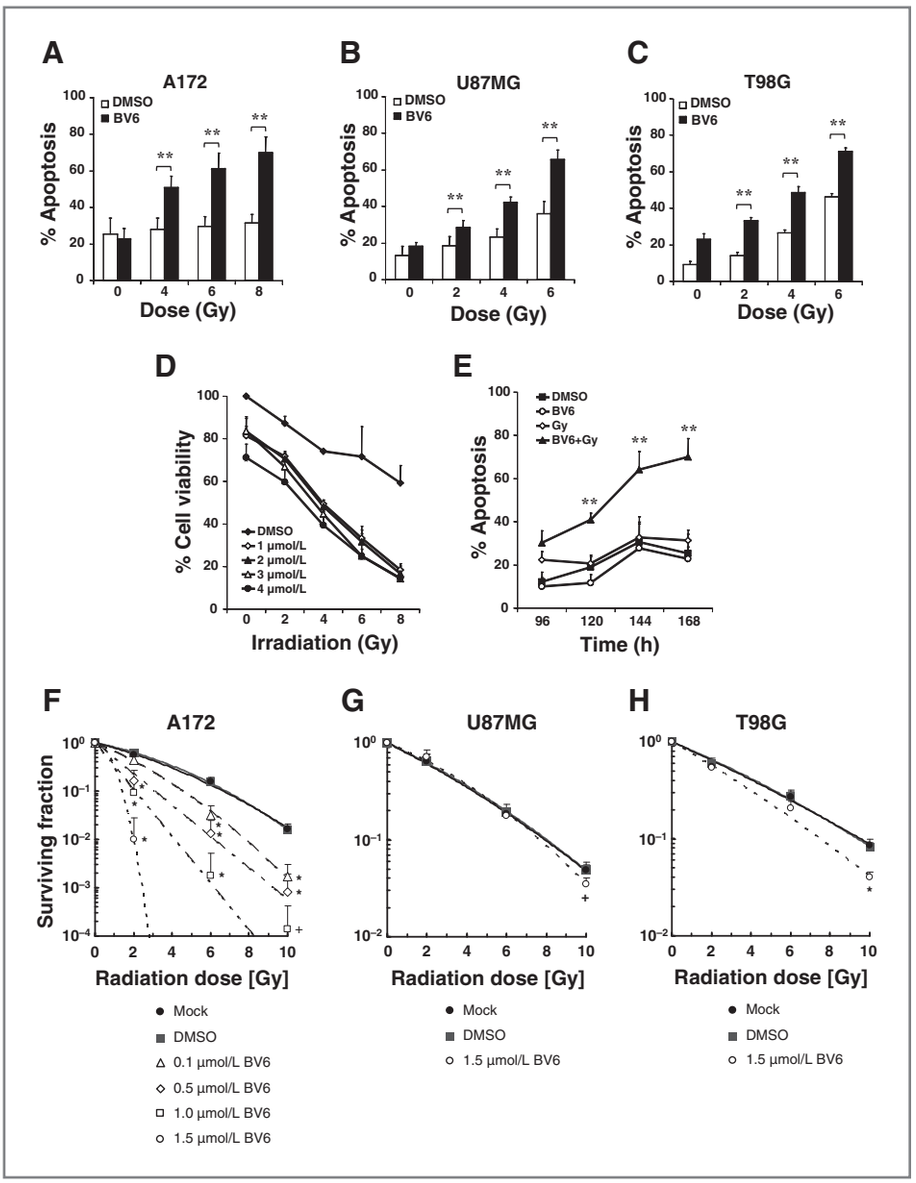

Figure 1. BV6 enhances γ-irradiation–induced apoptosis in glioblastoma cells. In A–C, glioblastoma cells were treated for 168 hours (A172, U87MG) or 144 hours (T98G) with indicated doses of γ-irradiation and/or 2 μmol/L BV6 (A172, T98G) or 3 μmol/L BV6 (U87MG). In D, A172 cells were treated with indicated doses of γ-irradiation and/or indicated concentrations of BV6 for 168 hours. In D, A172 cells were treated with indicated doses of γ-irradiation and/or indicated concentrations of BV6 for 168 hours. In E, A172 cells were treated with 8 Gy γ-irradiation and/or 2 μmol/L BV6 for indicated times. In A–C, E, apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide–stained nuclei. In D, cell viability was determined by MTT assay and is expressed as percentage of untreated controls. Data are mean + SEM of 3 independent experiments conducted in triplicate; **, $P < 0.001$. In E, combined treatment with BV6 and radiation was compared with radiation treatment alone. In F–H, clonogenic colony formation assay was done as described in Materials and Methods, and the surviving fraction after 11 to 14 days is shown. Data are mean + SD of at least 3 independent experiments; +, $P < 0.05$; *, $P < 0.01$.

significantly reduced BV6- and TNFα-induced apoptosis that was used as a positive control (Fig. 2B and C). This indicates that BV6 and γ-irradiation induce apoptosis independently of an autocrine/paracrine TNFα loop.

Identification of NF-κB as a critical mediator of BV6-mediated sensitization for γ-irradiation–induced apoptosis

Next, we investigated whether modulation of NF-κB activity by BV6 is critical for radiosensitization, as IAP proteins are involved in the regulation of NF-κB signaling (9). Treatment with BV6 stimulated NF-κB DNA binding, whereas the combination treatment with BV6 and γ-irradiation resulted in reduced NF-κB DNA binding compared with treatment with BV6 alone (Fig. 3A). Stimulation of NF-κB transcriptional activity by BV6 was confirmed by luciferase reporter assay (Supplementary Fig. S3). To examine the composition of the NF-κB subunits, we conducted supershift analysis. A prominent shift was found upon the addition of p50 antibody, although some reduction of NF-κB DNA binding was observed upon the addition of RelB, cRel, and p52 antibodies (Fig. 3B). This indicates that the DNA-binding complex upon combined treatment with BV6 and γ-irradiation predominately consists of the NF-κB subunit p50.

To investigate the functional role of NF-κB in this model of apoptosis, we used glioblastoma cell lines, in which NF-κB activation is inhibited by overexpression of IκBα superrepressor (IκBα-SR; ref. 24). This dominant-negative

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Table 1. Radiation response of glioblastoma cells upon treatment with BV6 and radiation

Cell line treatment	Plating efficiency [%]	α [Gy^{-1}]	β [Gy^{-2}]	LD_{50} [Gy]	Radiation enhancement ratio	LD_{10} [Gy]	Radiation enhancement ratio
A172							
Mock treated	32.4	0.2274	0.0178	2.54		6.66	
DMSO treated	28.6	0.1813	0.0232	2.81		6.79	
BV6 [1.5 μmol/L]	1.1	0.0000	1.1488	0.78	3.62	1.42	4.80
T98G							
Mock treated	15.7	0.2142	0.0029	3.11		9.53	
DMSO treated	16.0	0.1984	0.0050	3.23		9.39	
BV6 [1.5 μmol/L]	6.2	0.2738	0.0045	2.43	1.33	7.49	1.25
U87MG							
Mock treated	14.2	0.2371	0.0067	2.72		7.94	
DMSO treated	13.9	0.2224	0.0080	2.83		8.03	
BV6 [1.5 μmol/L]	8.0	0.1905	0.0146	2.96	0.95	7.63	1.05

NOTE: Radiation enhancement ratios at 50% (LD_{50}) and 10% survival (LD_{10}) were calculated by transforming the linear quadratic equation ($SF = \exp[-\alpha \times D - \beta \times D^2]$) using α and β values of the individual survival curves.

form of IκBα harbors 2 point mutations and therefore cannot be phosphorylated and degraded, thus preventing NF-κB translocation to the nucleus and subsequent NF-κB activation. Importantly, inhibition of NF-κB prevented the BV6-mediated sensitization for γ-irradiation–induced apoptosis in different glioblastoma cell lines (Fig. 3C–E), showing that NF-κB exerts a proapoptotic function in this model of apoptosis.

To investigate whether NF-κB regulates apoptosis by affecting the Smac mimetic–triggered downregulation of IAP proteins, we determined expression levels of IAP proteins by Western blotting. Treatment with BV6 caused rapid and profound downregulation of cIAP1 both in cells overexpressing IκBα-SR and vector control (Supplementary Fig. S4), consistent with the current model that Smac mimetics stimulate autoubiquitination and proteasomal degradation of IAP proteins with a RING domain and E3 ligase activity (10–12). Also, XIAP protein expression decreased upon treatment with BV6 alone and with the combination of BV6 and γ-irradiation, both in cells overexpressing IκBα-SR and in vector control cells (Supplementary Fig. S1). These findings suggest that NF-κB–conferred radiosensitivity is not mediated by differential modulation of IAP proteins.

To further explore the regulation of apoptosis by NF-κB, we monitored activation of the caspase cascade and mitochondrial perturbations. Strikingly, NF-κB inhibition profoundly reduced activation of caspase-8 and -3 into active fragments (Fig. 4A). Also, the

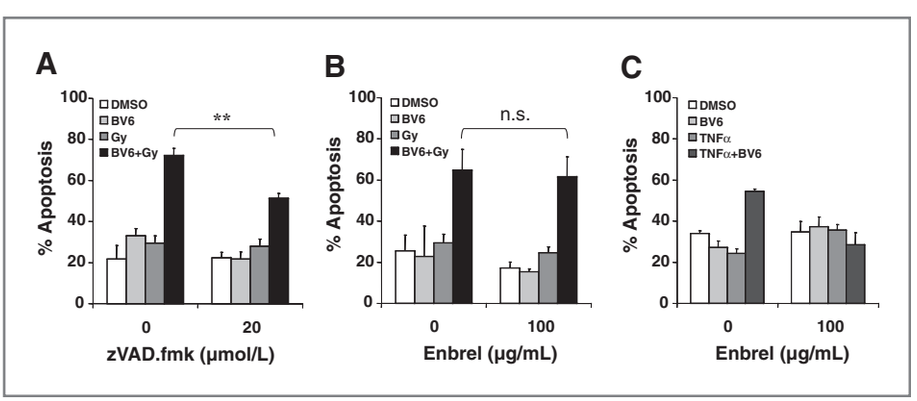

Figure 2. BV6-mediated sensitization to γ-irradiation–induced apoptosis is caspase dependent. A172 cells were treated for 168 hours with 8 Gy γ-irradiation and/or 2 μmol/L BV6 in the presence or absence of 20 μmol/L zVAD.fmk or 100 μg/mL Enbrel. Treatment with 10 ng/mL TNFα and 2 μmol/L BV6 for 48 hours served as a positive control for Enbrel. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide–stained nuclei. Data are mean + SEM of 3 (A, B) or 1 (C) independent experiments conducted in triplicate; **, $P < 0.001$ comparing BV6 with solvent; n.s., not significant.

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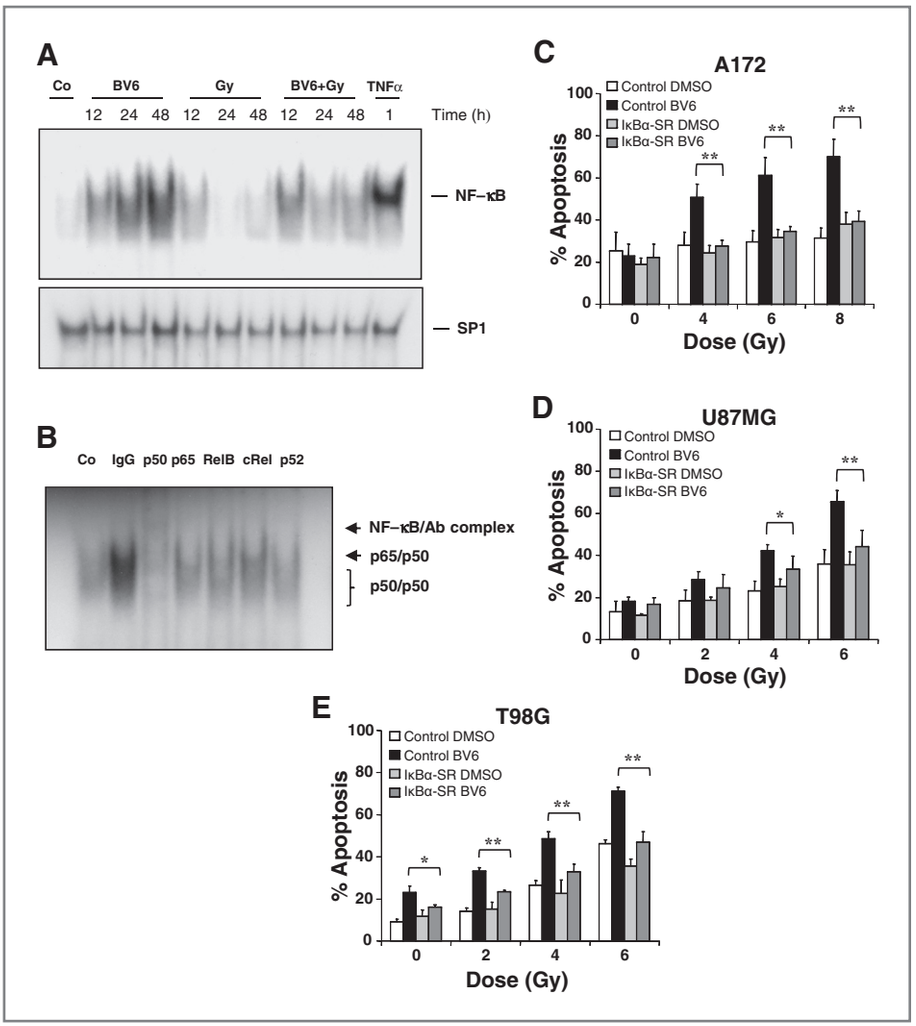

Figure 3. NF-κB activation is required for BV6-mediated sensitization to γ-irradiation–induced apoptosis. In A, A172 cells were left untreated (Co) or were treated with 8 Gy γ-irradiation and/or 2 μmol/L BV6 for indicated times. Stimulation with 10 ng/mL TNFα for 1 hour served as positive control. NF-κB activation was assessed by the analysis of NF-κB DNA binding by EMSA. One representative out of 3 experiments is shown. In B, NF-κB complex composition following treatment of A172 cells with 8 Gy γ-irradiation and/or 2 μmol/L BV6 for 12 hours was assessed by subjecting nuclear extracts to EMSA analysis with or without preincubation with specific antibodies against p50, p65, RelB, cRel or p52 or with IgG. In C–E, A172 (C), U87MG (D) or T98G (E) cells stably transduced with a vector containing IκBα-SR or empty control vector were treated with indicated doses of γ-irradiation and/or 2 μmol/L BV6 (A172, T98G) or 3 μmol/L BV6 (U87MG) for 168 hours (C, D) or 144 hours (E). Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide–stained nuclei. Data are mean + SEM of 3 independent experiments conducted in triplicate; *, $P < 0.005$; **, $P < 0.001$ comparing IκBα-SR overexpressing to control vector cells.

BV6- and γ-irradiation–induced loss of mitochondrial membrane potential and cytochrome *c* release into the cytosol were markedly decreased in cells overexpressing IκBα-SR compared with vector control cells (Fig. 4B and C).

NF-κB inhibition by kinase dead IKKβ abolishes BV6-mediated radiosensitization

To confirm the proapoptotic role of NF-κB in this model of apoptosis, we used an alternative approach to inhibit NF-κB activity by overexpression of a kinase dead version of IKKβ (IKKβ-KD). Expression levels of IKKβ-KD were controlled by Western blot analysis (Fig. 5A). EMSA confirmed that IKKβ-KD inhibits NF-κB activation upon TNFα stimulation that was used as a positive control for NF-κB activation (Fig. 5B). Importantly, NF-κB inhibition by IKKβ-KD significantly reduced apoptosis by the combination treatment with BV6 and γ-irradiation (Fig. 5C). These data confirm by an independent genetic approach that NF-κB promotes BV6- and γ-irradiation–induced apoptosis.

BV6 sensitizes primary cultured glioma cells and glioblastoma-initiating cancer stem cells for γ-irradiation

Finally, we extended our experiments to primary glioma samples obtained from surgical specimens to investigate the potential clinical relevance of BV6 for radiosensitization. Of note, BV6 significantly increased γ-irradiation–induced apoptosis in primary cultured glioma cells (Fig. 6A). Furthermore, we explored the effect of BV6 on the sensitivity of glioblastoma-initiating cancer stem cells toward γ-irradiation because they have been implicated in radioresistance (28). Glioblastoma-initiating cancer stem cells were isolated from clinical samples and were characterized as we previously described (17, 20) by CD133 and Nestin staining and by their ability to differentiate into the 3 different neuronal lineages (data not shown). Importantly, BV6 significantly increased

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γ-irradiation–induced apoptosis of glioblastoma-initiating cancer stem cells obtained from several specimens (Fig. 6B–D). These findings show that BV6 sensitizes primary cultured glioma cells as well as glioblastoma-initiating cancer stem cells for γ-irradiation.

Discussion

Because defects in apoptosis programs, for example, high expression of antiapoptotic molecules, can cause resistance to treatment regimens including radiotherapy (8), current attempts to improve the outcome of glioblastoma patients depend on strategies to increase apoptosis sensitivity. In this study, we identify a new proapoptotic role of NF-κB in γ-irradiation-mediated apoptosis of glioblastoma cells by showing, for the first time, that NF-κB is critically required for Smac mimetic–triggered radiosensitization. This conclusion is supported by several independent pieces of evidence. First, BV6 and γ-irradiation cooperate to trigger apoptosis in glioblastoma cells. This interaction is highly synergistic in A172 glioblastoma cells (CI < 0.3). Second, BV6 stimulates NF-κB activation, which is required for the potentiation of γ-irradiation–induced apoptosis because genetic inhibition of NF-κB by overexpression of the dominant-negative superrepressor IκBα-SR significantly reduces BV6- and γ-irradiation–induced apoptosis. Also, the BV6-conferred increase in γ-irradiation–induced caspase activation, loss of mitochondrial membrane potential, and cytochrome *c* release is abolished in cells overexpressing

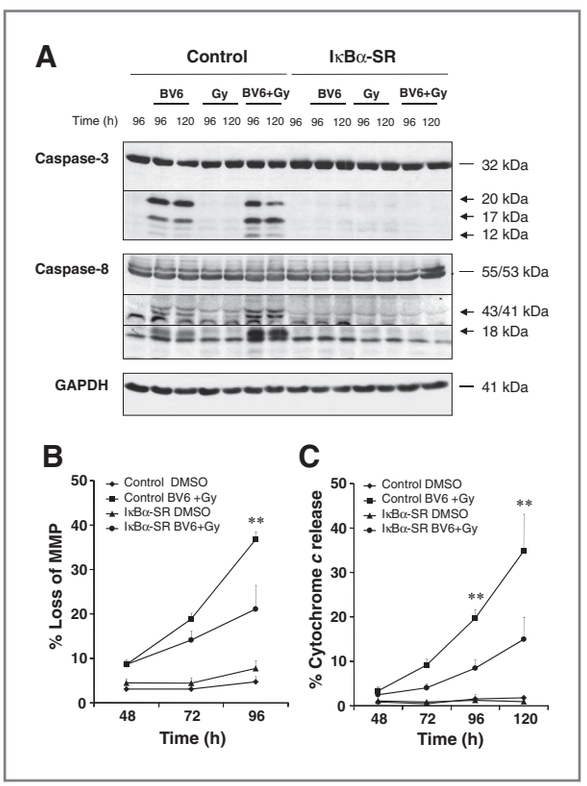

Figure 4. NF-κB inhibition by IκBα-SR prevents BV6-mediated sensitization to γ-irradiation–induced caspase activation and mitochondrial outer membrane permeabilization. A172 cells stably transduced with a vector containing IκBα-SR or empty control vector were treated with 2 μmol/L BV6 and/or 8 Gy γ-irradiation for indicated times. In A, cleavage of caspase-8 and -3 was assessed by Western blotting; cleavage fragments are indicated by arrowhead. GAPDH served as loading control. One representative of 2 experiments is shown. In B and C, mitochondrial transmembrane potential (B) and cytochrome *c* release (C) were assessed by FACS analysis. Data are mean + SEM of 3 independent experiments conducted in triplicate; **, $P < 0.001$ comparing IκBα-SR overexpressing with control vector cells following γ-irradiation.

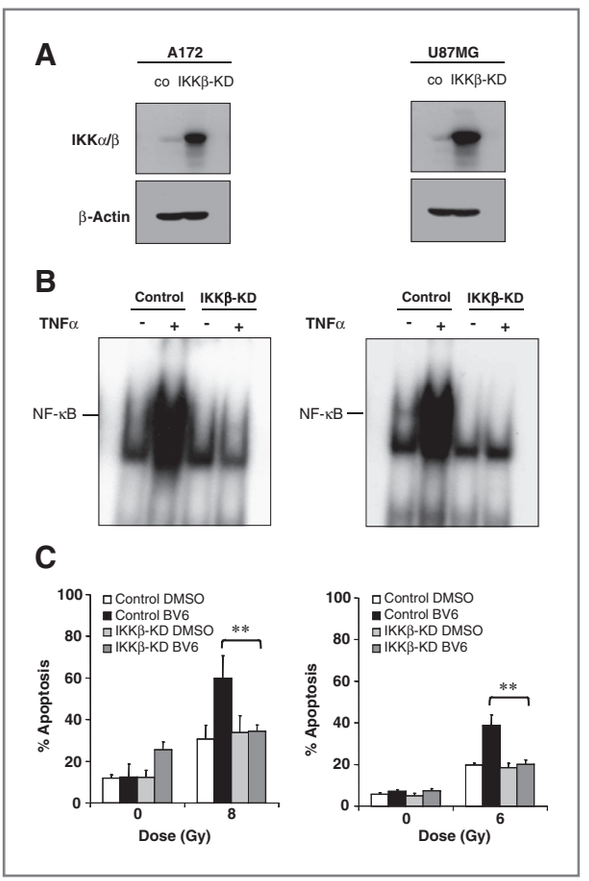

Figure 5. NF-κB inhibition by kinase dead IKKβ inhibits BV6-mediated radiosensitization. A, A172 (left) and U87MG (right) cells were stably transduced with a vector containing kinase dead IKKβ (IKKβ-KD) or empty control vector (co). Expression of IKKα/β was determined by Western blotting. In B, NF-κB activation in control and IKKβ-KD overexpressing cells was determined by EMSA after stimulation with 10 ng/mL TNFα for 1 hour. In C, IKKβ-KD overexpressing and control cells were treated with 2 μmol/L BV6 and/or 8 Gy (A172) or 6 Gy (U87MG) γ-irradiation for 168 hours. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide–stained nuclei. Data are mean + SEM of 3 independent experiments conducted in triplicate; **, $P < 0.001$ comparing IKKβ-KD overexpressing with control vector cells.

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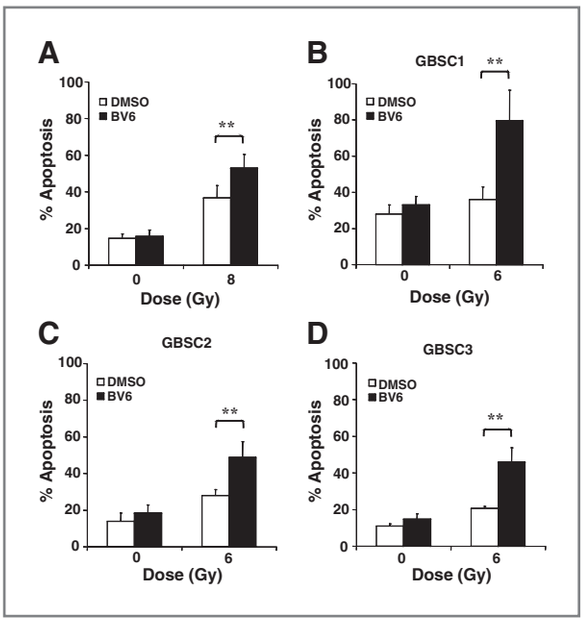

Figure 6. BV6 enhances γ-irradiation–induced apoptosis in primary cultured glioblastoma and glioblastoma-initiating cancer stem cells. In A, primary cultured glioblastoma cells were treated with 8 Gy γ-irradiation and/or 2 μmol/L BV6 for 168 hours. In B–D, glioblastoma-initiating cancer stem cells (GBSC1-3) were seeded at $0.05 \times 10^5/cm^2$ and treated with 6 Gy γ-irradiation and/or 6 μmol/L BV6 for 168 hours. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide–stained nuclei. Data are mean + SEM of 3 independent experiments conducted in triplicate; **, $P < 0.001$ comparing BV6 with solvent.

IκBα-SR. Similarly, NF-κB inhibition by ectopic expression of a kinase-dead form of IKKβ abolishes the BV6-mediated radiosensitization. Together, these findings provide clear evidence that NF-κB promotes apoptosis upon treatment with BV6 and γ-irradiation.

The novelty of this article, in particular, resides in the identification of NF-κB as a critical mediator of BV6-mediated sensitization for γ-irradiation–induced apoptosis. By comparison, TNFα turned out to be dispensable for BV6- and γ-irradiation–induced apoptosis because Enbrel, a TNFα blocking antibody, did not prevent cell death induction. TNFα has previously been implicated to mediate cell death induced by single agent treatment with Smac mimetic (10–12) or by combinations of Smac mimetic with chemotherapeutics (29, 30). This suggests that the involvement of TNFα in Smac mimetic-induced apoptosis is context dependent. It will be subject to future studies to identify the TNFα-independent mechanisms that mediate the proapoptotic function of NF-κB in the context of Smac mimetic–mediated radiosensitization in glioblastoma cells.

Furthermore, our study shows that NF-κB promotes, rather than inhibits, apoptosis in the context of γ-irradiation. This is particularly noteworthy in light of the fact that NF-κB is well known for its antiapoptotic function (13). More recently, NF-κB has also been reported to promote apoptosis under certain circumstances (31). A possible explanation for the observed proapoptotic function of NF-κB in this study is that NF-κB activation may lead to upregulation of proapoptotic target genes, which remains subject to future investigation. It is interesting to note that NF-κB has recently been reported to mediate the AF1q-induced upregulation of BAD, a proapoptotic member of the Bcl-2 family of proteins (32). We previously identified a novel proapoptotic role of NF-κB in the chemotherapy-stimulated DNA damage response in glioblastoma cells (24). Accordingly, topoisomerase II inhibitors that intercalate into the DNA such as doxorubicin, daunorubicin, and mitoxantrone trigger NF-κB activation before the induction of apoptosis, and NF-κB inhibition significantly reduces apoptosis (24). In addition, a proapoptotic function of NF-κB has been described in several systems of neuronal cell death, for example, induced by ischemia, glutamate, betulinic acid, or NMDA receptors stimulation (25, 33–36).

As far as cell death induced by single agent treatment with Smac mimetics is concerned, NF-κB has been reported either to promote or to inhibit apoptosis. Although inhibition of NF-κB decreases Smac mimetic–mediated cell death in ovarian carcinoma and rhabdomyosarcoma cells (10), NF-κB inhibition has been reported to enhance Smac mimetic–triggered cytotoxicity in lung or prostate carcinoma cells (37, 38). These findings point to a differential pro- or antiapoptotic role of NF-κB in Smac mimetic-induced cell death, possibly dependent on the cell type and/or cell line. Additional studies are required to unravel the underlying molecular mechanisms of NF-κB–mediated regulation of Smac mimetic–induced cell death.

Our results have important implications for the development of combination protocols with radiotherapy and Smac mimetic in the treatment of glioblastoma. Of note, BV6 also sensitizes primary cultured glioma cells derived from surgical specimens for γ-irradiation, underscoring the clinical relevance of our findings. Furthermore, BV6 significantly increased γ-irradiation–induced apoptosis in glioblastoma-initiating cancer stem cells, which have recently been accused of conferring radioresistance (28). In conclusion, Smac mimetics such as BV6 present a promising strategy for radiosensitization of glioblastoma, which warrants further investigation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. DeAngelis LM. Brain tumors. N Engl J Med 2001;344:114–23.
2. Stupp R, Hegi ME, Gilbert MR, Chakravarti A. Chemoradiotherapy in malignant glioma: standard of care and future directions. J Clin Oncol 2007;25:4127–36.
3. Chakravarti A, Palanichamy K. Overcoming therapeutic resistance in malignant gliomas: current practices and future directions. Cancer Treat Res 2008;139:173–89.
4. Lockshin RA, Zakeri Z. Cell death in health and disease. J Cell Mol Med 2007;11:1214–24.
5. Fulda S, Debatin KM. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene 2006;25:4798–811.
6. Kroemer G, Galluzzi L, Brenner C. Mitochondrial membrane permeabilization in cell death. Physiol Rev 2007;87:99–163.
7. Kögel D, Fulda S, Mittelbronn M. Therapeutic exploitation of apoptosis and autophagy for glioblastoma. Anticancer Agents Med Chem 2010;10:438–49.
8. Fulda S. Tumor resistance to apoptosis. Int J Cancer 2009;124:511–5.
9. LaCasse EC, Mahoney DJ, Cheung HH, Plenchette S, Baird S, Korneluk RG. IAP-targeted therapies for cancer. Oncogene 2008;27:6252–75.
10. Vince JE, Wong WW, Khan N, Feltham R, Chau D, Ahmed AU, et al. IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. Cell 2007;131:682–93.
11. Varfolomeev E, Blankenship JW, Wayson SM, Fedorova AV, Kayagaki N, Garg P, et al. IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. Cell 2007;131:669–81.
12. Petersen SL, Wang L, Yalcin-Chin A, Li L, Peyton M, Minna J, et al. Autocrine TNFalpha signaling renders human cancer cells susceptible to Smac-mimetic-induced apoptosis. Cancer Cell 2007;12:445–56.
13. Baud V, Karin M. Is NF-kappaB a good target for cancer therapy? Hopes and pitfalls. Nat Rev Drug Discov 2009;8:33–40.
14. Fulda S, Wick W, Weller M, Debatin KM. Smac agonists sensitize for Apo2L/TRAIL- or anticancer drug-induced apoptosis and induce regression of malignant glioma *in vivo*. Nat Med 2002;8:808–15.
15. Giagkousiklidis S, Vogler M, Westhoff MA, Kasperczyk H, Debatin KM, Fulda S. Sensitization for gamma-irradiation-induced apoptosis by second mitochondria-derived activator of caspase. Cancer Res 2005;65:10502–13.
16. Giagkousiklidis S, Vellanki SH, Debatin KM, Fulda S. Sensitization of pancreatic carcinoma cells for gamma-irradiation-induced apoptosis by XIAP inhibition. Oncogene 2007;26:7006–16.
17. Vellanki SH, Grabrucker A, Liebau S, Proepper C, Eramo A, Braun V, et al. Small-molecule XIAP inhibitors enhance gamma-irradiation-induced apoptosis in glioblastoma. Neoplasia 2009;11:743–52.
18. Opel D, Westhoff MA, Bender A, Braun V, Debatin KM, Fulda S. Phosphatidylinositol 3-kinase inhibition broadly sensitizes glioblastoma cells to death receptor- and drug-induced apoptosis. Cancer Res 2008;68:6271–80.
19. Ishii N, Maier D, Merlo A, Tada M, Sawamura Y, Diserens AC, et al. Frequent co-alterations of TP53, p16/CDKN2A, p14ARF, PTEN tumor suppressor genes in human glioma cell lines. Brain Pathol 1999;9:469–79.
20. Eramo A, Ricci-Vitiani L, Zeuner A, Pallini R, Lotti F, Sette G, et al. Chemotherapy resistance of glioblastoma stem cells. Cell Death Differ 2006;13:1238–41.
21. Fulda S, Sieverts H, Friesen C, Herr I, Debatin KM. The CD95 (APO-1/Fas) system mediates drug-induced apoptosis in neuroblastoma cells. Cancer Res 1997;57:3823–9.
22. Rodel C, Haas J, Groth A, Grabenbauer GG, Sauer R, Rodel F. Spontaneous and radiation-induced apoptosis in colorectal carcinoma cells with different intrinsic radiosensitivities: survivin as a radioresistance factor. Int J Radiat Oncol Biol Phys 2003;55:1341–7.
23. Mohr A, Zwacka RM, Debatin KM, Stahnke K. A novel method for the combined flow cytometric analysis of cell cycle and cytochrome c release. Cell Death Differ 2004;11:1153–4.
24. Karl S, Pritschow Y, Volcic M, Hacker S, Baumann B, Wiesmuller L, et al. Identification of a novel pro-apopotic function of NF-kappaB in the DNA damage response. J Cell Mol Med 2009;13:4239–56.
25. Kasperczyk H, La Ferla-Bruhl K, Westhoff MA, Behrend L, Zwacka RM, Debatin K-M, et al. Betulinic acid as new activator of NF-kappaB: molecular mechanisms and implications for cancer therapy. Oncogene 2005;24:6945–56.
26. Chou TC. The median-effect principle and the combination index for quantitation of synergism and antagonism. In: Chou TC, editor. Synergism and antagonism in chemotherapy. San Diego, USA: Academic Press; 1991. p. 61–102.
27. Wang L, Du F, Wang X. TNF-alpha induces two distinct caspase-8 activation pathways. Cell 2008;133:693–703.
28. Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature 2006;444:756–60.
29. Dineen SP, Roland CL, Greer R, Carbon JG, Toombs JE, Gupta P, et al. Smac mimetic increases chemotherapy response and improves survival in mice with pancreatic cancer. Cancer Res 2010;70:2852–61.
30. Probst BL, Liu L, Ramesh V, Li L, Sun H, Minna JD, et al. Smac mimetics increase cancer cell response to chemotherapeutics in a TNF-alpha-dependent manner. Cell Death Differ 2010;17:1645–54.
31. Radhakrishnan SK, Kamalakaran S. Pro-apoptotic role of NF-kappaB: implications for cancer therapy. Biochim Biophys Acta 2006;1766:53–62.
32. Co NN, Tsang WP, Tsang TY, Yeung CL, Yau PL, Kong SK, et al. AF1q enhancement of gamma irradiation-induced apoptosis by up-regulation of BAD expression via NF-kappaB in human squamous carcinoma A431 cells. Oncol Rep 2010;24:547–54.
33. Mattson MP. NF-kappaB in the survival and plasticity of neurons. Neurochem Res 2005;30:883–93.
34. Herrmann O, Baumann B, de Lorenzi R, Muhammad S, Zhang W, Kleesiek J, et al. IKK mediates ischemia-induced neuronal death. Nat Med 2005;11:1322–9.
35. Pizzi M, Goffi F, Boroni F, Benarese M, Perkins SE, Liou HC, et al. Opposing roles for NF-kappa B/Rel factors p65 and c-Rel in the modulation of neuron survival elicited by glutamate and interleukin-1beta. J Biol Chem 2002;277:20717–23.
36. Qin ZH, Chen RW, Wang Y, Nakai M, Chuang DM, Chase TN. Nuclear factor kappaB nuclear translocation upregulates c-Myc and p53 expression during NMDA receptor-mediated apoptosis in rat striatum. J Neurosci 1999;19:4023–33.
37. Bai L, Chen W, Wang X, Tang H, Lin Y. IKKbeta-mediated nuclear factor-kappaB activation attenuates smac mimetic-induced apoptosis in cancer cells. Mol Cancer Ther 2009;8:1636–45.
38. Petersen SL, Wang L, Yalcin-Chin A, Li L, Peyton M, Minna J, et al. Autocrine TNFalpha signaling renders human cancer cells susceptible to Smac-mimetic-induced apoptosis. Cancer Cell 2007;12:445–56.

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