NF-κB is required for Smac mimetic-mediated sensitization of glioblastoma cells for γ-irradiation-induced apoptosis

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Abbreviations: BIR, baculovirus IAP repeat; CI, combination index; cIAP1, cellular inhibitor of apoptosis 1; DIABLO, direct IAP binding protein with low pI; FCS, fetal calf serum; IAP, Inhibitor of apoptosis; IκBα-SR, IκBα superrepressor; IKK, IκB kinase complex; IKKβ-KD, kinase dead IKKβ; Smac, second mitochondria-derived activator of caspase; TMRE, Tetramethylrhodamine methylester perchlorate; XIAP, X-linked inhibitor of apoptosis; zVAD.fmk, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

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Abstract

Evasion of apoptosis contributes to radioresistance of glioblastoma, calling for novel strategies to overcome apoptosis resistance. In the present study, we investigated the potential of the small molecule Smac mimetic BV6 to modulate radiosensitivity of glioblastoma cells. Here, we identify a novel pro-apoptotic function of NF-κB in γ-irradiation-induced apoptosis of glioblastoma cells by demonstrating 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 value <0.3). Molecular studies show that BV6 stimulates NF-κB activation, which is critical for radiosensitization, since 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.
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 in order to improve the dismal prognosis of this cancer (3).

Apoptosis is the cell’s intrinsic death program 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 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, while Smac/DIABLO promotes apoptosis by neutralizing ‘Inhibitor of Apoptosis’ (IAP) proteins (6).

Evasion of apoptosis is one of the hallmarks of human cancers including glioblastoma (7) and also contributes to chemoresistance or radioresistance, since 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 anti-apoptotic proteins (8), for example by high levels of ‘Inhibitor of apoptosis’ (IAP) proteins (9). IAP proteins all harbor a baculovirus IAP repeat (BIR) domain, while 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 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 nuclear factor-kappaB (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 anti-apoptotic 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 demonstrated that genetic or pharmacological inactivation of XIAP increases radiation-induced apoptosis in glioblastoma, neuroblastoma and pancreatic carcinoma cells (15-17). In order 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 the present 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 (Manassas, VA) that performs cell line authentication by short tandem repeat analysis. Cells were cultured in DMEM (Life Technologies, Inc., Eggenstein, Germany) supplemented with 10% fetal calf serum (FCS) (Biochrom, Berlin, Germany), 1 mM glutamine (Biochrom), 1% penicillin/streptavidin (Biochrom) and 25 mM 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) (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 EGF and 10 μg/ml bFGF 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 (Suppl. Fig. 1) (11). BV6 was kindly provided by Genentech Inc. (South San Francisco, CA). All chemicals were purchased from Sigma (Steinheim, Germany) unless indicated otherwise. N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk) was obtained from Bachem (Heidelberg, Germany), Enbrel from Pfizer (New York, NY).

Determination of apoptosis

Cells were treated with γ-irradiation (Nuclear Data, CS-137, 44Tbq, 2Gy/min, Frankfurt, Germany) at indicated doses and incubated for indicated times in the presence of BV6 or 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, Heidelberg, Germany) of DNA fragmentation of propidium iodide-stained nuclei as described (21).

**Clonogenic survival assay**

The clonogenic colony formation assay was performed 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 µM), 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, 10 Gy) using a linear accelerator (SL 75/5; Elekta, Crawley, UK) with 6 Megaelectron Volt (MEV) photons/100 cm focus-surface distance with a dose rate of 4.0 Gy/min. After 11-14 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 x (PE / 100), taking into consideration the individual plating efficiency (PE). Survival variables $\alpha$ and $\beta$ 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 three times. Radiation enhancement ratios at 50% and 10% survival were calculated by transforming the above mentioned equation using $\alpha$ and $\beta$ values of the individual survival curves.

**Determination of mitochondrial membrane potential and cytochrome c release**

Tetramethylrhodamine methylester perchlorate (TMRE; 1µM; 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 anti-cytochrome c monoclonal antibody (BD Biosciences) as described (23).
Western blot analysis

Western blot analysis was performed as described previously (18) using the following antibodies: mouse anti-caspase-8 (ApoTech Corporation, Epalinges, Switzerland), rabbit anti-caspase-3 (Cell Signaling, Beverly, MA), mouse anti-XIAP from BD Biosciences, goat anti-cIAP1 from R&D Systems, Inc. (Wiesbaden, Germany), mouse anti-β-actin (Sigma) or mouse anti-GAPDH (HyTest, Turku, Finland) followed by goat-anti-mouse or goat-anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). Enhanced chemiluminescence was used for detection (Amersham Bioscience, Freiburg, Germany).

Retroviral Transduction

Retroviral transduction of dominant-negative IκBα superrepressor (IκBα-SR) or kinase dead IKKβ (IKKβ-KD) was performed as previously described (24) using the pCFG5-IEGZ retroviral vector system. In brief, PT67 producer cells (Clontech, Palo Alto, CA) were transfected with pCFG5-IEGZ vectors using Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s recommendation and selected with 0.25 mg/ml Zeocin (Invivogen, San Diego, CA). 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 (EMSA)

Nuclear extracts were prepared as previously described (25). In brief, cells were washed, scraped and collected by centrifugation at 1000 x 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, Rockford, IL,
USA). For EMSA, the following oligomers were used: NF-κB 5'-AGTTGAGGGGACTTTCCCAGGC-3' (sense), Sp1- 5'-ATTGCATCGGGCAGAGGAG-3' (sense). Single-stranded oligonucleotides were labeled with γ-[^32P]-ATP by T4-polynucleotide kinase (MBI Fermentas GmbH, St. Leon-Rot, Germany), annealed and purified on sephadex columns (Micro Bio-Spin P30, Biorad Laboratories, Munich, Germany). Binding reactions containing 5 µg nuclear extract, 1 µg Poly(dI:dC) (Sigma), labeled oligonucleotide (10,000 cpm) and 5x binding buffer were incubated on ice and resolved by electrophoresis in non-denaturing 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 (Santa Cruz Biotechnology). Representative EMSAs are shown.

**Statistical analysis**

Statistical significance was assessed by Student's t-test using Winstat software (R. Fitch Software, Bad Krozingen, Germany). Drug interactions were analyzed by the Combination index (CI) method based on that described by Chou (26) using CalcuSyn software (Biosoft, Cambridge, UK). Combination index (CI) <0.9 indicates synergism, <0.3 high synergism, 0.9-1.1 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 mimic 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 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 (Suppl. Fig. 2A). Calculation of combination index revealed that BV6 cooperated in a highly synergistic manner with γ-irradiation to trigger apoptosis (Fig. 1D, suppl. Tab. 1). 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 performed colony assays. We selected four concentrations of BV6 for these experiments (0.1-1.5 μM 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 (Tab. 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 two cell lines. This indicates that BV6 markedly increases radiation-induced apoptosis in all three glioblastoma cell lines, while 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 (Suppl. Fig. 2B). Together, this set of experiments demonstrates 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 pharmacological 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), demonstrating 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, while it significantly reduced BV6- and TNFα-induced apoptosis that was used as a positive control (Fig. 2B, C). This indicates that BV6 and γ-irradiation induce apoptosis independent 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, while the combination treatment with BV6 and γ-irradiation resulted in reduced NF-κB DNA binding compared to treatment with BV6 alone (Fig. 3A). Stimulation of NF-κB transcriptional activity by BV6 was confirmed by luciferase reporter assay (Suppl. Fig. 3). To examine the composition of the NF-κB subunits, we performed supershift analysis. A prominent shift was found upon the addition of p50 antibody, while 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) (24). This dominant-negative form of IκBα harbors two 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), demonstrating that NF-κB exerts a pro-apoptotic 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 (suppl. Fig. 4) 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 (suppl. Fig. 1). 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 BV6- and γ-irradiation-induced loss of mitochondrial membrane potential (MMP) and cytochrome c
release into the cytosol were markedly decreased in cells overexpressing IκBα-SR compared to vector control cells (Fig. 4B, C).

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

To confirm the pro-apoptotic role of NF-κB in this model of apoptosis, we employed 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 towards γ-irradiation, since 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 three different neuronal lineages (data not shown). Importantly, BV6 significantly increased γ-irradiation-induced apoptosis of glioblastoma-initiating cancer stem cells obtained from several specimens (Fig.
6B-D). These findings demonstrate that BV6 sensitizes primary cultured glioma cells as well as glioblastoma-initiating cancer stem cells for γ-irradiation.
Discussion

Since defects in apoptosis programs, for example high expression of anti-apoptotic 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 the present study, we identify a new pro-apoptotic 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, since 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 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 the current report 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, since 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 pro-apoptotic function of NF-κB in the context of Smac mimetic-mediated radiosensitization in glioblastoma cells.

Furthermore, our study demonstrates 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 anti-apoptotic function (13). More recently, NF-κB has also been reported to promote apoptosis under certain circumstances (31). A possible explanation for the observed pro-apoptotic function of NF-κB in the present study is that NF-κB activation may lead to upregulation of pro-apoptotic 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 pro-apoptotic member of the Bcl-2 family of proteins (32). We previously identified a novel pro-apoptotic 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 prior to the induction of apoptosis and NF-κB inhibition significantly reduces apoptosis (24). In addition, a pro-apoptotic function of NF-κB has been described in several systems of neuronal cell death, e.g. 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. While 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 anti-apoptotic 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 to confer radioresistance (28). In conclusion, Smac mimetics such as BV6 present a promising strategy for radiosensitization of glioblastoma, which warrants further investigation.

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References


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

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Plating efficiency [%]</th>
<th>$\alpha$ [Gy$^{-1}$]</th>
<th>$\beta$ [Gy$^2$]</th>
<th>$LD_{50}$ [Gy]</th>
<th>Radiation enhancement ratio</th>
<th>$LD_{10}$ [Gy]</th>
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<td>A172</td>
<td>32.4</td>
<td>0.2274</td>
<td>0.0178</td>
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<td>6.88</td>
<td>6.79</td>
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<td>DMSO-treated</td>
<td>28.6</td>
<td>0.1813</td>
<td>0.0232</td>
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<td>BV6 [1.5 µM]</td>
<td>1.1</td>
<td>0.0000</td>
<td>1.4868</td>
<td>0.78</td>
<td>3.62</td>
<td>1.42</td>
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<tr>
<td>T98G</td>
<td>15.7</td>
<td>0.2142</td>
<td>0.0029</td>
<td>3.11</td>
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<td>9.39</td>
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<td>0.0050</td>
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<td>BV6 [1.5 µM]</td>
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<td>2.96</td>
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Radiation enhancement ratios at 50% ($LD_{50}$) and 10% survival ($LD_{10}$) were calculated by transforming the linear quadratic equation ($SF = \exp \left(-\alpha x D - \beta x D^2\right)$) using $\alpha$ and $\beta$ values of the individual survival curves.
Figure legends

**Fig. 1. BV6 enhances γ-irradiation-induced apoptosis in glioblastoma cells.**

In (a-c), Glioblastoma cells were treated for 168h (A172, U87MG) or 144h (T98G) with indicated doses of γ-irradiation and/or 2 µM BV6 (A172, T98G) or 3 µM BV6 (U87MG). In (d), A172 cells were treated with indicated doses of γ-irradiation and/or indicated concentrations of BV6 for 168h. In (d), A172 cells were treated with indicated doses of γ-irradiation and/or indicated concentrations of BV6 for 168h. In (e), A172 cells were treated with 8 Gy γ-irradiation and/or 2 µM 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 three independent experiments performed in triplicate; **, \( P < 0.001 \).

**Fig. 2. BV6-mediated sensitization to γ-irradiation-induced apoptosis is caspase-dependent.**

A172 cells were treated for 168h with 8 Gy γ-irradiation and/or 2 µM BV6 in the presence or absence of 20 µM zVAD.fmk or 100 µg/ml Enbrel. Treatment with 10 ng/ml TNFα and 2 µM BV6 for 48h served as a positive control for Enbrel. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei. Data are mean + SD of at least three independent experiments; + \( P < 0.05 \); * \( P < 0.01 \).
Fig. 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 μM BV6 for indicated times. Stimulation with 10 ng/ml TNFα for 1h served as positive control. NF-κB activation was assessed by the analysis of NF-κB DNA binding by EMSA. One representative out of three experiments is shown. In (b), NF-κB complex composition following treatment of A172 cells with 8 Gy γ-irradiation and/or 2 μM BV6 for 12h 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 μM BV6 (A172, T98G) or 3 μM BV6 (U87MG) for 168h (c, d) or 144h (e). Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei. Data are mean + SEM of three independent experiments performed in triplicate; *, P<0.005; **, P<0.001 comparing IκBα-SR overexpressing to control vector cells.

Fig. 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 μM 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 out of two 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 three independent experiments performed in triplicate; **, \( P < 0.001 \) comparing IkBα-SR overexpressing to control vector cells following γ-irradiation.

**Fig. 5. NF-κB inhibition by kinase dead IKKβ inhibits BV6-mediated radiosensitization.**

A172 (left panels) and U87MG (right panels) cells were stably transduced with a vector containing kinase dead IKKβ (IKKβ-KD) or empty control vector (co). In (a), 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 1h. In (c), IKKβ-KD overexpressing and control cells were treated with 2 μM BV6 and/or 8 Gy (A172) or 6 Gy (U87MG) γ-irradiation for 168h. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei. Data are mean + SEM of three independent experiments performed in triplicate; **, \( P < 0.001 \) comparing IKKβ-KD overexpressing to control vector cells.

**Fig. 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 μM BV6 for 168h. In (b-d), glioblastoma-initiating cancer stem cells (GBSC1-3) were seeded at 0.05 x 10⁵/cm² and treated with 6 Gy γ-irradiation and/or 6 μM BV6 for 168 h. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei. Data are mean + SEM of three independent experiments performed in triplicate; **, \( P < 0.001 \) comparing BV6 to solvent.
Figure 1

(a) A172

(b) U87MG

(c) T98G

(d) % cell viability

(e) % apoptosis

(f) Survival fraction

- Mock
- DMSO
- 0.1 µM BV6
- 0.5 µM BV6
- 1.0 µM BV6
- 1.5 µM BV6
Figure 2

a

![Graph showing % apoptosis vs zVAD.fm (µM) with treatments DMSO, BV6, Gy, and BV6+Gy.](image)

** n.s. between 0 and 20 µM zVAD.fm

b

![Graph showing % apoptosis vs Enbrel (µg/ml) with treatments DMSO, BV6, Gy, and BV6+Gy.](image)

n.s. between 0 and 100 µg/ml Enbrel

c

![Graph showing % apoptosis vs Enbrel (µg/ml) with treatments DMSO, BV6, TNFα, TNFα+BV6.](image)

* significant difference between 0 and 100 µg/ml Enbrel
Figure 3

Panel a: Gel electrophoresis showing bands for NF-κB and SP1.

Panel b: Gel electrophoresis showing bands for NF-κB/Ab complex, p65/p50, and p50/p50.

Panel c: Bar graph showing % apoptosis over time (h) for A172 cells.

Panel d: Bar graph showing % apoptosis over dose (Gy) for U87MG cells.

Panel e: Bar graph showing % apoptosis over dose (Gy) for T98G cells.
Figure 4

a

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>IkBα-SR</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV6</td>
<td>Gy</td>
<td>BV6+Gy</td>
</tr>
<tr>
<td>Time (h)</td>
<td>96 96 120</td>
<td>96 120 120</td>
</tr>
</tbody>
</table>

Caspase-3

- 32 kD
- 20 kD
- 17 kD
- 12 kD

Caspase-8

- 55/53 kD
- 43/41 kD
- 18 kD

GAPDH

- 41 kD

b

- control DMSO
- control BV6 +Gy
- IkBα-SR DMSO
- IkBα-SR BV6+Gy

% loss of MMP

Time (h)

48 72 96

% cytochrome c release

Time (h)

48 72 96 120

**
Figure 5

a

A172

co IKKβ-KD

IKKα/β

β-actin

U87MG

co IKKβ-KD

b

control IKKβ-KD

TNFα - + - +

NF-κB

control IKKβ-KD

TNFα - + - +

NF-κB

c

% apoptosis

control DMSO
control BV6
IKKβ-KD DMSO
IKKβ-KD BV6

% apoptosis

control DMSO
control BV6
IKKβ-KD DMSO
IKKβ-KD BV6

Dose (Gy)

0 8

Dose (Gy)

0 6

**
Figure 6

a

% apoptosis

0 20 40 60 80 100

Dose (Gy)

GBSC1

DMSO

BV6

b

% apoptosis

0 20 40 60 80 100

Dose (Gy)

GBSC1

DMSO

BV6

C

% apoptosis

0 20 40 60 80 100

Dose (Gy)

GBSC2

DMSO

BV6

d

% apoptosis

0 20 40 60 80 100

Dose (Gy)

GBSC3

DMSO

BV6 6µM
Molecular Cancer Therapeutics

NF-κB is required for Smac mimetic-mediated sensitization of glioblastoma cells for γ-irradiation-induced apoptosis

Rebecca Berger, Claudia Jennewein, Sabine Karl, et al.

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