NEOPLASIA
www.neoplasia.com

Volume 13 Number 12 December 2011 pp. 1162–1170 1162

Requirement of Nuclear Factor κB for Smac Mimetic–Mediated Sensitization of Pancreatic Carcinoma Cells for Gemcitabine-Induced Apoptosis[1,2]

Dominic Stadel*, Silvia Cristofanon†, Behnaz Ahangarian Abhari†, Kurt Deshayes‡, Kerry Zobel‡, Domagoj Vucic‡, Klaus-Michael Debatin* and Simone Fulda*,†

*University Children's Hospital, Ulm University, Ulm, Germany; †Institute for Experimental Cancer Research in Pediatrics, Goethe-University, Frankfurt, Germany; ‡Genentech, Inc, South San Francisco, CA, USA

Abstract

Defects in apoptosis contribute to treatment resistance and poor outcome of pancreatic cancer, calling for novel therapeutic strategies. Here, we provide the first evidence that nuclear factor (NF) κB is required for Smac mimetic–mediated sensitization of pancreatic carcinoma cells for gemcitabine-induced apoptosis. The Smac mimetic BV6 cooperates with gemcitabine to reduce cell viability and to induce apoptosis. In addition, BV6 significantly enhances the cytotoxicity of several anticancer drugs against pancreatic carcinoma cells, including doxorubicin, cisplatin, and 5-fluorouracil. Molecular studies reveal that BV6 stimulates NF-κB activation, which is further increased in the presence of gemcitabine. Importantly, inhibition of NF-κB by overexpression of the dominant-negative IκBα superrepressor significantly decreases BV6- and gemcitabine-induced apoptosis, demonstrating that NF-κB exerts a proapoptotic function in this model of apoptosis. In support of this notion, inhibition of tumor necrosis factor α (TNFα) by the TNFα blocking antibody Enbrel reduces BV6- and gemcitabine-induced activation of caspase 8 and 3, loss of mitochondrial membrane potential, and apoptosis. By demonstrating that BV6 and gemcitabine trigger a NF-κB–dependent, TNFα-mediated loop to activate apoptosis signaling pathways and caspase-dependent apoptotic cell death, our findings have important implications for the development of Smac mimetic–based combination protocols in the treatment of pancreatic cancer.

Neoplasia (2011) 13, 1162–1170

Introduction

Pancreatic cancer belongs to the leading causes of cancer deaths in the Western world [1]. Treatment resistance of pancreatic cancer, for example, to chemotherapy, remains a major challenge in oncology, and this can be caused by evasion of apoptosis—the cell's intrinsic cell death program [2]. This highlights the need for novel strategies to overcome apoptosis resistance in pancreatic cancer.

Apoptosis signaling pathways operate through two major routes, i.e., through the death receptor (extrinsic) pathway and through the mitochondrial (intrinsic) pathway, which result in activation of caspases as common effector molecules of cell death [3]. Activation of receptors of the tumor necrosis factor (TNF) receptor superfamily, for example, TNF-related apoptosis-inducing ligand (TRAIL) receptors or TNF receptor 1 (TNFR1), results in activation of the initiator caspase 8, which in turn activates effector caspases such as caspase 3 [4]. The intrinsic (mitochondrial) pathway involves the permeabilization of the outer mitochondrial membrane and the release of mitochondrial

Abbreviations: BIR, baculovirus IAP repeat; cIAP1, cellular inhibitor of apoptosis 1; IAP, inhibitor of apoptosis; IκBα-SR, IκBα superrepressor; Smac, second mitochondria-derived activator of caspase; XIAP, X-linked inhibitor of apoptosis; zVAD.fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

Address all correspondence to: Prof. Dr. Simone Fulda, Institute for Experimental Cancer Research in Pediatrics, Goethe-University Frankfurt, Komturstr. 3a, 60528 Frankfurt, Germany. E-mail: simone.fulda@kgu.de

[1]This work has been partially supported by grants from the Deutsche Forschungsgemeinschaft, the European Community (ApopTrain, APO-SYS) and IAP6/18 (to S.F.). The authors declare that they do not have any conflict of interest.

[2]This article refers to supplementary materials, which are designated by Figures W1 to W6 and are available online at www.neoplasia.com.

Received 30 March 2011; Revised 5 November 2011; Accepted 8 November 2011

DOI 10.1593/neo.11460

intermembrane space proteins such as cytochrome *c* and second mitochondria-derived activator of caspase (Smac)/direct inhibitor of apoptosis (IAP) binding protein with low p*I* into the cytosol [5]. Cytochrome *c* triggers caspase 3 activation through the apoptosome complex, whereas Smac promotes caspase 3 activation by binding to and neutralizing X-linked IAP (XIAP) [5].

IAP proteins comprise eight individual members that all harbor a baculovirus IAP repeat (BIR) domain [6]. In addition, XIAP, cellular IAP 1 (cIAP1), and cIAP2 harbor a RING domain with E3 ubiquitin ligase activity, which mediates (auto)ubiquitination and proteasomal degradation [6]. XIAP is best characterized for its antiapoptotic function by binding to and inhibiting caspase 9 and caspase 3/7 through its BIR3 domain and the linker region preceding BIR2 domain, respectively [6]. Recently, cIAP1 and cIAP2 were identified as E3 ubiquitin ligases for the serine/threonine kinase RIP1 that put K63-linked ubiquitin chains on RIP1 [7,8]. Furthermore, a cIAP-TRAF destruction complex keeps the basal level of NIK low and is involved in regulating noncanonical NF-κB signaling [6]. In addition to neutralizing the inhibitory function of XIAP on caspase activation, Smac mimetics have been shown to trigger autoubiquitination and proteasomal degradation of IAP proteins with a RING domain, thereby promoting NF-κB activation and TNFα-dependent cell death [9–11].

The transcription factor NF-κB functions as a dimer that is composed of proteins of the NF-κB/Rel family [12]. On stimulation, the IκB kinase complex becomes activated, which initiates the proteasomal degradation of IκBα, which in turn releases NF-κB to translocate to the nucleus [12]. NF-κB is usually considered to negatively regulate apoptosis, for example, through transcriptional activation of antiapoptotic proteins [12].

We previously reported that inhibition of XIAP profoundly enhances TRAIL-induced apoptosis in pancreatic carcinoma *in vitro* and *in vivo* [13–15]. Searching for novel strategies to enhance chemosensitivity of pancreatic cancer, we investigated the effect of a small molecule Smac mimetic on anticancer drug–induced apoptosis in the present study.

Materials and Methods

Cell Culture and Reagents

Pancreatic carcinoma cells were cultured in Dulbecco modified Eagle medium (Life Technologies, Inc, Eggenstein, Germany) supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany), 1 mM glutamine (Biochrom), 1% penicillin/streptavidin (Biochrom), and 25 mM HEPES (Biochrom) as described [15]. The bivalent Smac mimetic BV6 has previously been characterized, and the structure of the compound (Figure W1) has previously been published [10]. Gemcitabine was obtained from Lilly (Bad Homburg, Germany); doxorubicin, etoposide, and cisplatin were obtained from Sigma (Steinheim, Germany); *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk) was obtained from Bachem (Heidelberg, Germany); Enbrel was obtained from Pfizer (New York, NY); and necrostatin-1 was obtained from Biomol (Hamburg, Germany). All chemicals were purchased from Sigma unless indicated otherwise.

Determination of Apoptosis and Cell Viability

Apoptosis was determined by fluorescence-activated cell-sorting analysis (FACScan; BD Biosciences, Heidelberg, Germany) of DNA fragmentation of propidium iodide–stained nuclei as previously described [16]. Cell viability was assessed by MTT assay according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany).

Western Blot Analysis

Western blot analysis was performed as described [15] using the following antibodies: mouse anti–caspase 8 (ApoTech Corporation, Epalinges, Switzerland), rabbit anti–caspase 3 (Cell Signaling, Beverly, MA), mouse anti-XIAP, mouse anti–Bcl-X_L from BD Biosciences, rabbit anti-cIAP2 (Epitomics, Burlingame, CA), goat anti-cIAP1 and rabbit anti-survivin (R&D Systems, Inc, Wiesbaden, Germany), rabbit anti-IκBα (Santa Cruz Biotechnology, Santa Cruz, CA), or mouse anti–β-actin (Sigma) followed by goat-antimouse IgG or goat-antirabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology). Enhanced chemiluminescence was used for detection (Amersham Bioscience, Freiburg, Germany).

Determination of Mitochondrial Membrane Potential and Cytochrome c Release

For determination of mitochondrial transmembrane, potential cells were incubated with tetramethylrhodamine methylester perchlorate (0.2 μg/ml; Sigma) for 10 minutes at 37°C and immediately analyzed by flow cytometry.

Retroviral Transduction

Overexpression of the dominant-negative IκBα superrepressor was performed by retroviral transduction using IκBα (S32; 36A) and the pCFG5-IEGZ retroviral vector system as previously described [17]. In brief, stable PT67 producer cells (Clontech, Palo Alto, CA) were transfected with empty pCFG5-IEGZ vectors or pCFG5-IEGZ vectors containing IκBα (S32; 36A) using Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) according to the manufacturer's recommendation and selected with 0.25 mg/ml Zeocin (InvivoGen, San Diego, CA). Stable Panc1 cells 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 [18]. In brief, cells were washed, scraped, and collected by centrifugation at 1000*g* for 5 minutes at 4°C. Cells were resuspended in low-salt buffer and allowed to swell on ice for 12 minutes; a 10% Igepal CA-630 (Sigma-Aldrich) solution was added and after vortexing the cell suspension was centrifuged again. The pelleted nuclei were resuspended in high-salt buffer, incubated on ice, and vortexed at times for 20 minutes. Nuclear supernatants were obtained by centrifugation at 12,500*g* at 4°C for 12 minutes. Protein concentrations were determined using the BCA Protein assay Kit (Pierce, Rockford, IL). For electrophoretic mobility shift assay (EMSA), the following sequence was used as specific oligomer for NF-κB: 5′-AGTTGAGGGGACTTTCCCAGGC-3′ (sense). Single-stranded oligonucleotides were labeled with γ-[^{32}P]-ATP by T4-polynucleotide kinase (MBI Fermentas GmbH, St. Leon-Rot, Germany), annealed to the complementary oligomer strand, and purified on sephadex columns (Micro Bio-Spin P30; Bio-Rad Laboratories, Munich, Germany). Binding reactions containing 5 μg of nuclear extract, 1 μg of poly(dI:dC) (Sigma), labeled oligonucleotide (10,000 cpm), and 5× binding buffer were incubated for 30 minutes on ice. Binding complexes were resolved by electrophoresis in nondenaturing 6% polyacrylamide gels using 0.3× TBE as running buffer and assessed by autoradiography. Representative EMSAs are shown.

Luciferase Assay

The Dual-Luciferase Reporter Assay System (Promega, Madison, WI) was used to determine firefly and *Renilla* luciferase activities as previously described [18] using 3× κB-firefly luciferase vector containing 3× κB consensus motif (CCCTGAAAGG) or a *Renilla* luciferase vector under the control of the ubiquitin promoter. Firefly luciferase values were normalized to *Renilla* luciferase values.

EMSA

EMSA was performed with IRDye-labeled double-stranded oligonucleotides containing a NF-κB consensus site (5′-AGTTGAGGGGACTTTCCCAGGC-3′) or NF-κB mutated site (5′-AGTTGAATTCACTTTCCCAGGC-3′) using the Odyssey infrared imaging system (LI-COR, Bad Homburg, Germany) according to the instructions of the manufacturer. The DNA binding reactions were set up by incubating

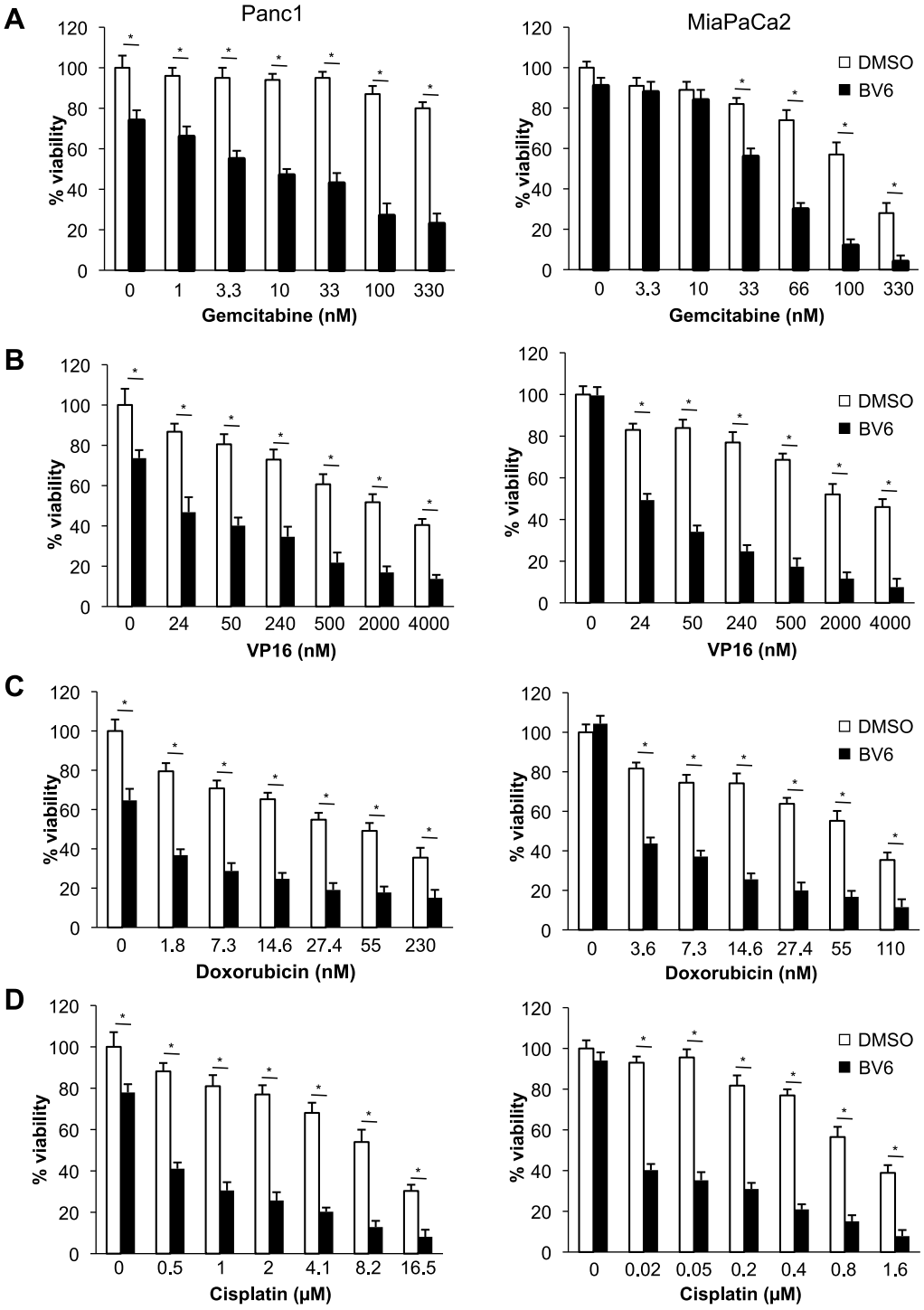

Figure 1. BV6 enhanced gemcitabine-induced loss of viability. Panc1 (left) and MiaPaCa2 (right) cells were treated with indicated concentrations of gemcitabine (A), etoposide (B), doxorubicin (C), and cisplatin (D) and/or 2 μM BV6 for 72 hours. Cell viability was assessed by MTT assay and is calculated as percentage of untreated cells. Means ± SEM of three independent experiments performed in triplicate are shown. *$P < .01$ comparing samples in the presence or absence of BV6.

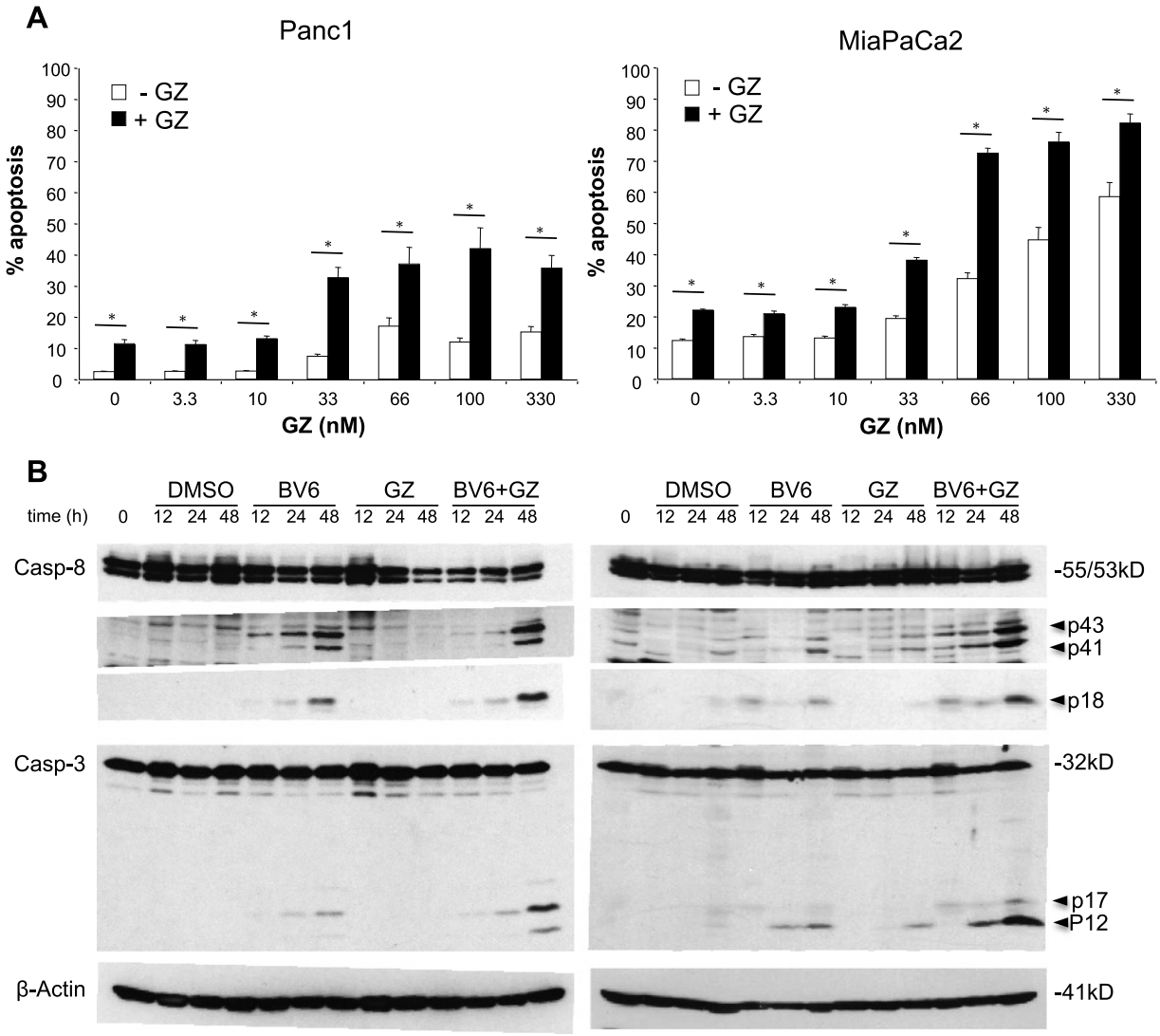

Figure 2. BV6 enhances gemcitabine-induced apoptosis. (A) DNA fragmentation. Panc1 (left) and MiaPaCa2 (right) cells were treated with indicated concentrations of gemcitabine and/or 2 μM BV6 for 72 hours. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide–stained nuclei. Means ± SEM of three independent experiments performed in triplicate are shown. *$P < .01$ comparing samples in the presence or absence of BV6. (B) Caspase cleavage. Panc1 (left) and MiaPaCa2 (right) cells were treated with 100 nM (Panc1) or 66 nM (MiaPaCa2) gemcitabine and/or 2 μM BV6 for indicated time points. Cleavage of caspase 8 and 3 was assessed by Western blot analysis. β-Actin served as loading control. One representative Western blot of two independent experiments is shown.

15 μg of nuclear proteins and 125 fmol of oligonucleotides in the dark for 30 minutes at room temperature in 20 μl of binding buffer (20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.25 mg/ml polydeoxyinosinic-polydeoxycytidylic acid). The reaction mixture was run on a 5% PAGE gel in high ionic strength buffer (0.05 M Tris, pH 8.5, 380 mM glycine, 2 mM EDTA). Gels were recorded using the Odyssey infrared imaging system (LI-COR). Binding of labeled oligonucleotides was shown to be sequence-specific by demonstrating that binding was blocked by an excess (250 fmol) of unlabeled oligonucleotides.

Cell Cycle Analysis

Cell cycle analysis was performed by propidium iodide staining of permeabilized cells and flow cytometry (FACSCanto II; Becton Dickinson, Heidelberg, Germany). A total of 10,000 events were counted for each sample. Data were analyzed with FlowJo software (Celeza GmbH, Olten, Switzerland) choosing the Dean-Jet-Fox model analysis.

Determination of TNFα Secretion

TNFα levels in cell supernatants were determined by ELISA (BD Biosciences) according to the manufacturer's instructions.

Statistical Analysis

Statistical significance was assessed by two-sided Student's *t* test using Microsoft Excel (Microsoft Deutschland GmbH, Unterschleißheim, Germany).

Results

BV6 Promotes Chemotherapy-Induced Apoptosis

To investigate whether the bivalent Smac mimetic BV6 alters chemosensitivity of pancreatic carcinoma cells, we analyzed the effect of several chemotherapeutic drugs on cell viability in the presence and absence of BV6. For these experiments, we chose a concentration of

BV6 that exerted limited cytotoxicity as single agent (Figure W2*A*). Importantly, the addition of BV6 significantly decreased viability in a dose-dependent manner on treatment of Panc1 and MiaPaCa2 pancreatic carcinoma cells with several different cytotoxic drugs, namely, gemcitabine, doxorubicin, cisplatin, and 5-fluorouracil, compared to treatment with either drug alone (Figure 1). Kinetic studies demonstrated the time dependency of the BV6-mediated sensitization (Figure W2*B*). This shows that the Smac mimetic BV6 sensitizes pancreatic carcinoma cells toward chemotherapy.

Since gemcitabine is the first-line chemotherapeutic agent that is used in the clinic for the treatment of pancreatic cancer, we focused subsequent experiments on this cytotoxic drug. To explore whether loss of viability was due to the induction of apoptosis, we determined DNA fragmentation as a characteristic marker of apoptosis. BV6 significantly enhanced gemcitabine-induced apoptosis as assessed by DNA fragmentation in a dose-dependent manner (Figure 2*A*). As an additional parameter of apoptotic cell death, we monitored caspase activation by Western blot analysis. Combination treatment with BV6 and gemcitabine resulted in enhanced cleavage of caspase 3 and 8 into active fragments compared to either single-agent treatment alone as evident from the increased processing of caspase 8 to the p43/p41 and p18 fragments and of caspase 3 to the p17/p12 active fragments

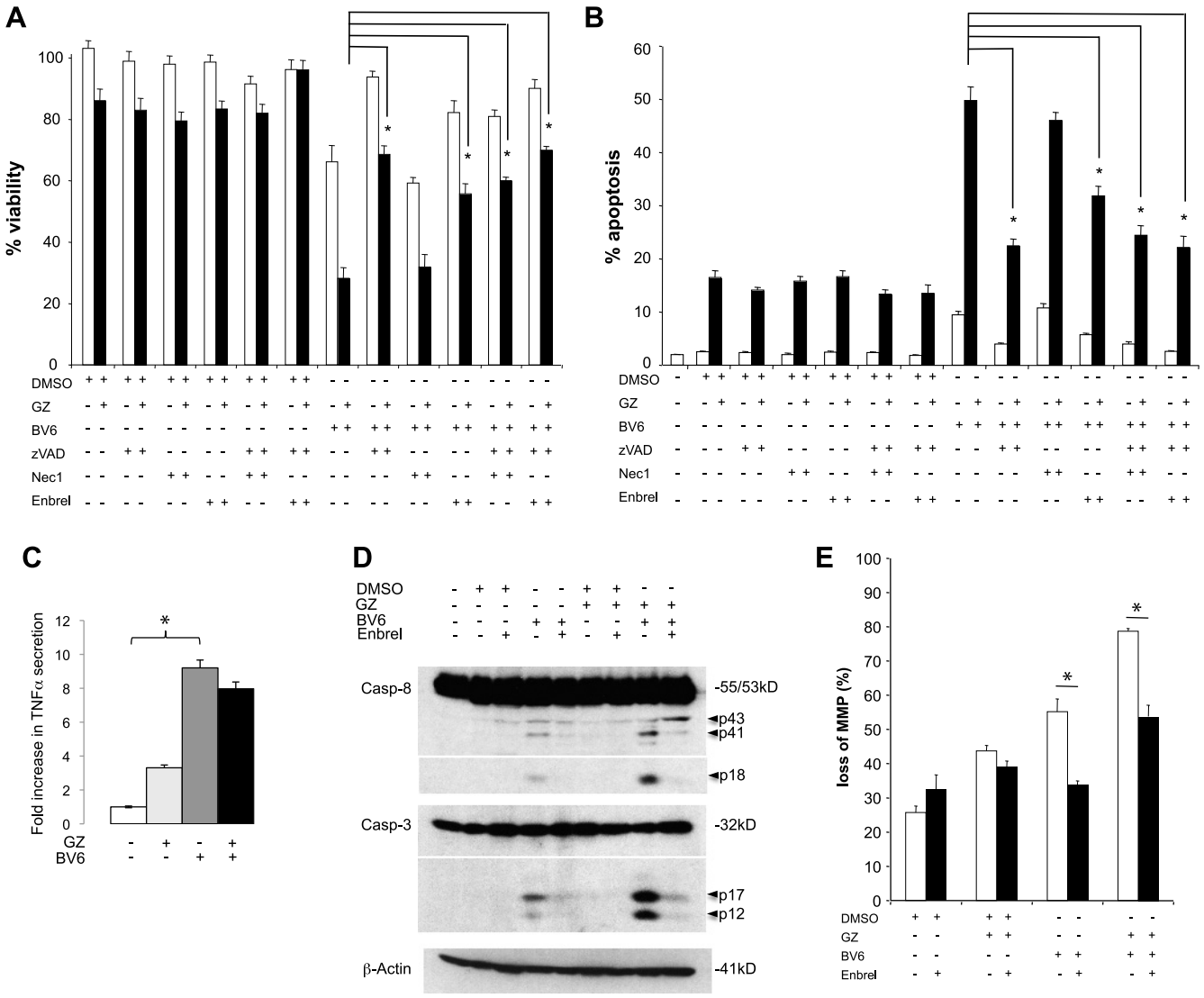

Figure 3. BV6- and gemcitabine-induced apoptosis is caspase- and TNFα-dependent. (A, B) Panc1 cells were treated for 72 hours with 100 nM gemcitabine and/or 2 μM BV6 and/or 20 μM zVAD.fmk, 30 μM necrostatin 1, 5 μg/ml Enbrel. Cell viability was assessed by MTT assay (A). Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide–stained nuclei (B). Means ± SEM of three independent experiments performed in triplicate are shown. *$P < .01$. (C) Panc1 cells were treated for 48 hours with 100 nM gemcitabine and/or 2 μM BV6. TNFα protein levels in supernatants were determined by ELISA, and fold increase in TNFα secretion relative to untreated control is shown. Means ± SD of three experiments performed in triplicate are shown. *$P < .05$ comparing BV6 treatment to untreated control. (D) Panc1 cells were treated 100 nM gemcitabine and/or 2 μM BV6 in the presence or absence of 5 μg/ml Enbrel for 48 hours. Cleavage of caspase 8 and 3 was assessed by Western blot analysis. One representative of two experiments is shown. (E) Panc1 cells were treated with 100 nM gemcitabine and/or 2 μM BV6 in the presence or absence of 5 μg/ml Enbrel for 48 hours. Loss of mitochondrial membrane potential (MMP) was assessed by flow cytometry. Means ± SEM of three independent experiments performed in triplicate are shown. *$P < .01$.

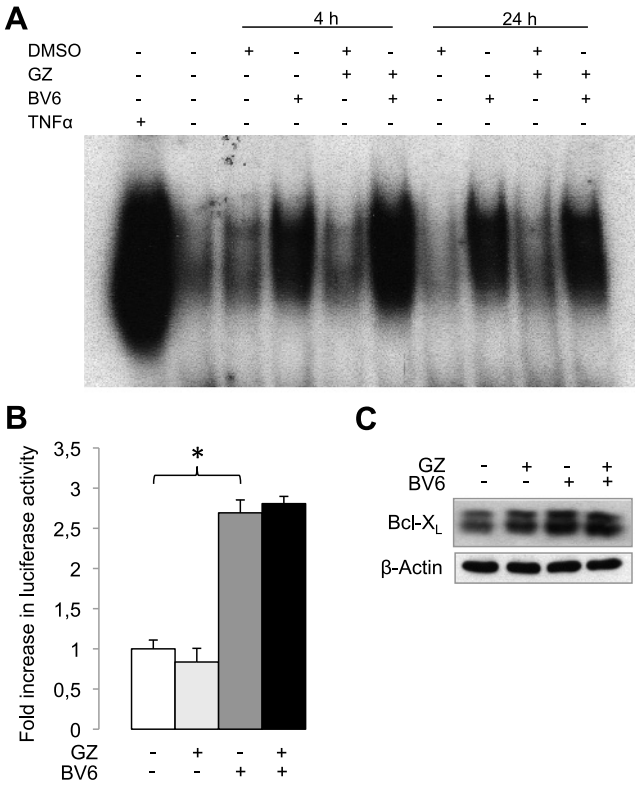

Figure 4. NF-κB activation by BV6. (A) Panc1 cells were treated with 100 nM gemcitabine and/or 2 μM BV6 for 4 or 24 hours. Stimulation with 10 ng/ml TNFα for 1 hour served as positive control. NF-κB activation was assessed by the analysis of NF-κB DNA binding by EMSA. One representative of three experiments is shown. (B) Panc1 cells were transiently transfected with firefly and *Renilla* luciferase gene constructs, treated with 100 nM gemcitabine and/or 2 μM BV6 for 24 hours, and analyzed by dual luciferase assay for induction of NF-κB transcriptional activity. Fold increase in luciferase activity relative to untreated control is shown. Means ± SD of three experiments performed in triplicate are shown. *$P < .05$ comparing BV6 treatment to untreated control. (C) Panc1 cells were treated with 100 nM gemcitabine and/or 2 μM BV6 for 24 hours. Protein expression of Bcl-X_L was assessed by Western blot analysis. β-Actin served as loading control.

(Figure 2*B*). Together, these results demonstrate that BV6 increases gemcitabine-induced caspase activation and apoptosis in pancreatic carcinoma cells.

Requirement of Caspases and TNFα for BV6- and Gemcitabine-Induced Apoptosis

To gain insight into the pathways involved in the BV6-mediated sensitization to gemcitabine, we analyzed the effect of BV6 on expression of IAP proteins. Treatment with BV6 caused rapid down-regulation of cIAP1 and cIAP2 (Figure W3). Also, the expression of XIAP was reduced on longer treatment with BV6 (MiaPaCa2) or the combination of BV6 and gemcitabine (Panc1 and MiaPaCa2; Figure W4). These findings are consistent with the reported proteasomal degradation of IAP proteins on treatment with Smac mimetics such as BV6 [10]. By comparison, the addition of BV6 did not alter gemcitabine-induced cell cycle changes (Figure W4).

Next, we tested the effect of different pharmacological inhibitors that interfere with individual pathways. The addition of the broad-range caspase inhibitor zVAD.fmk significantly rescued loss of viability and induction of apoptosis on the combination treatment with BV6 and gemcitabine (Figure 3, *A* and *B*), thus pointing to caspase-dependent cell death.

In addition, we tested the involvement of RIP1 and TNFα, which have been implicated in BV6-induced cell death [7,9–11,19]. Treatment with necrostatin 1, a RIP1-specific inhibitor [20], had no effect on BV6- and gemcitabine-induced loss of viability or induction of apoptosis (Figure 3, *A* and *B*). Of note, the TNFα antagonistic antibody Enbrel significantly reduced loss of viability and apoptosis induction on the combination treatment with BV6 and gemcitabine (Figure 3, *A* and *B*). To determine whether BV6 triggers TNFα release, we assessed TNFα in the supernatant. Indeed, treatment with BV6 or BV6 plus gemcitabine stimulated TNFα release (Figure 3*C*). This indicates that TNFα is involved in mediating BV6- and gemcitabine-induced cell death in an autocrine/paracine manner. The combined use of zVAD.fmk together with TNFα or necrostatin 1 did not augment the protective effect of zVAD.fmk alone (Figure 3, *A* and *B*), further supporting the notion that cell death occurs in a caspase-dependent manner.

Since TNF receptor activation after binding of TNFα can initiate both survival and cell death pathways, we next assessed the effect of Enbrel on the extrinsic and intrinsic apoptosis signaling pathways on treatment with BV6 and/or gemcitabine. For the extrinsic apoptosis pathway, we monitored activation of the caspase cascade. Importantly, cleavage of the initiator caspase 8 into active fragments after treatment with BV6 or BV6 plus gemcitabine was profoundly inhibited in the presence of Enbrel (Figure 3*D*). Similarly, Enbrel inhibited BV6- and gemcitabine-induced processing of the effector caspase 3 into active fragments (Figure 3*D*). We also explored whether activation of the intrinsic pathway was regulated by TNFα. Of note, Enbrel significantly reduced loss of mitochondrial membrane potential on treatment with BV6 as single agent and in combination with gemcitabine (Figure 3*E*). Together, this set of experiments suggests that TNFα mediates at least in part BV6- and gemcitabine-induced cell death.

NF-κB Promotes BV6- and Gemcitabine-Induced Apoptosis

As Smac mimetic–induced NF-κB activation has been reported to stimulate TNFα production [9–11], we next analyzed NF-κB DNA binding activity by EMSA. Treatment with BV6 stimulated NF-κB DNA binding, which was further increased transiently after the addition of gemcitabine for 4 hours (Figure 4*A*). By comparison, treatment with gemcitabine alone for up to 48 hours did not substantially alter NF-κB DNA binding (Figure 4*A* and data not shown). Stimulation with TNFα in the presence or absence of cold or mutated oligonucleotides was used as control for NF-κB activation (Figure W5). NF-κB transcriptional activation was confirmed by luciferase reporter assay (Figure 4*B*). To explore whether NF-κB activation results in up-regulation of anti-apoptotic genes, we performed Western blot analysis. Treatment with BV6-triggered down-regulation of cIAP1 and cIAP2 (Figure W3) was consistent with Smac mimetic–stimulated proteasomal degradation of IAP proteins [10], whereas it caused a slight increase in Bcl-X_L expression (Figure 4*C*).

To explore whether NF-κB inhibits or promotes cell death in this model of apoptosis, we blocked NF-κB translocation to the nucleus and subsequent NF-κB activation by overexpression of IκBα super-repressor (IκBα-SR), a dominant-negative form of IκBα that cannot be phosphorylated and degraded due to two point mutations. Control experiments demonstrated that IκBα-SR protein is expressed and inhibits NF-κB DNA binding on stimulation with TNFα, which

was used as a prototypic activator of NF-κB, or on stimulation with BV6 and/or gemcitabine (Figures 5, *A* and *B*, and W6). Importantly, inhibition of NF-κB profoundly inhibited loss of viability on treatment with BV6 and gemcitabine (Figure 5*C*). Similarly, NF-κB inhibition significantly reduced BV6- and BV6/gemcitabine–induced apoptosis (Figure 5*D*). These results demonstrate that NF-κB is critical for BV6- and gemcitabine-induced apoptosis.

Discussion

Pancreatic carcinoma represents a prototypic apoptosis-resistant cancer [2] calling for new concepts to activate the apoptotic machinery. In the present study, we provide the first evidence that the Smac mimetic BV6 sensitizes pancreatic carcinoma cells for gemcitabine-induced apoptosis in a NF-κB–dependent manner. The following independent pieces of evidence support this conclusion: First, BV6 primes pancreatic carcinoma cells for gemcitabine-mediated apoptosis and also significantly enhances the cytotoxicity of several anticancer drugs, including doxorubicin, cisplatin, and 5-fluorouracil. Second, inhibition of caspases by the broad-range inhibitor zVAD.fmk profoundly inhibits BV6- and gemcitabine-induced apoptosis, demonstrating that cell death occurs in a caspase-dependent manner. Third, BV6 stimulates NF-κB activation, which is further increased in the presence of gemcitabine. Fourth, inhibition of NF-κB by overexpression of the dominant-negative IκBα-SR significantly decreases BV6- and

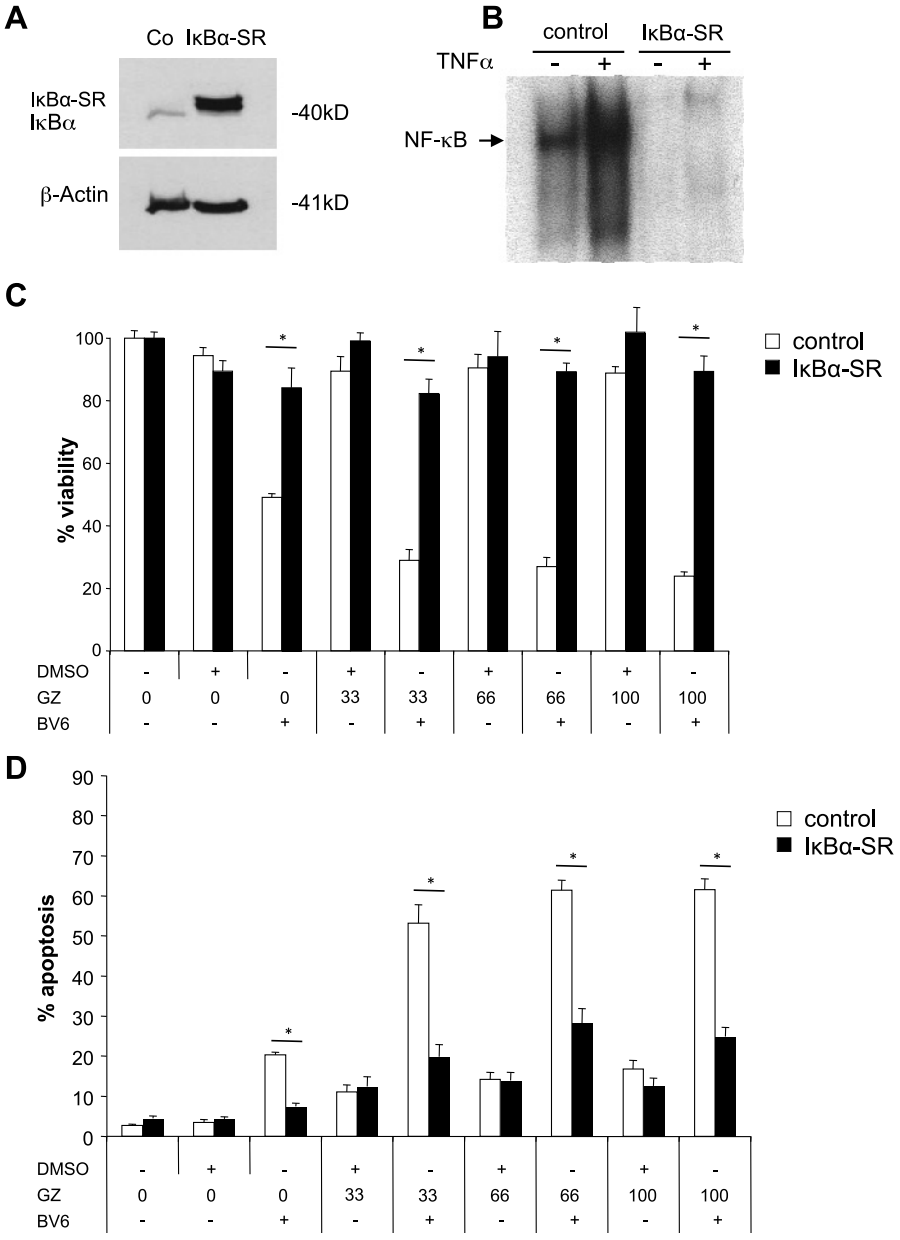

Figure 5. Requirement of NF-κB for BV6- and gemcitabine-induced cell death. Panc1 cells were stably transduced with a vector containing IκBα-SR or empty control vector. (A) Expression of IκBα-SR was determined by Western blot analysis using. (B) NF-κB activation in control and IκBα-SR–overexpressing cells was determined by EMSA after stimulation with 10 ng/ml TNFα for 1 hour. (C) IκBα-SR–overexpressing and control cells were treated with 2 μM BV6 and/or indicated concentrations of gemcitabine (nM) for 72 hours. Cell viability was determined by MTT assay, and this is calculated as the percentage of untreated cells. Means ± SEM of three independent experiments performed in triplicate are shown. *$P < .01$.

gemcitabine-induced apoptosis, pointing to a proapoptotic function of NF-κB in this model of apoptosis. Fifth, inhibition of TNFα by the TNFα blocking antibody Enbrel reduces activation of caspase 8 and 3, mitochondrial perturbation and apoptosis. These findings are consistent with a model that BV6 and gemcitabine trigger a NF-κB–dependent, TNFα-mediated loop to activate apoptosis signaling pathways and caspase-dependent apoptotic cell death.

Compared with our previous studies on the synergistic interaction of small molecule IAP inhibitors and the death receptor ligand TRAIL in pancreatic carcinoma [13,14,21], the novelty of the current report in particular resides in the demonstration that NF-κB is critically required for the BV6-mediated sensitization for gemcitabine-induced apoptosis. The proapoptotic function of NF-κB during apoptosis on treatment with BV6 alone or the combination of BV6 plus gemcitabine may be explained by the BV6-conferred switch of the TNFα response from survival to cell death through BV6-triggered degradation of cIAP1. Accordingly, Smac mimetics have been shown to interfere with cIAP-mediated ubiquitination of RIP1 by stimulating degradation of cIAPs, which favors the formation of the RIP1/FADD/caspase 8 death complex on stimulation of TNFR1 [7]. Consistently, we found that BV6- and gemcitabine-induced caspase activation, loss of mitochondrial membrane potential, and apoptosis occur in a TNFα-dependent manner because addition of the TNFα blocking antibody Enbrel inhibits all these events.

The identified proapoptotic function of NF-κB in the context of BV6- and chemotherapy-mediated apoptosis in pancreatic cancer cells is particularly noteworthy in light of the described antiapoptotic role of NF-κB in this type of cancer. To this end, NF-κB activation has been reported to prevent apoptosis in pancreatic carcinogenesis [22] and to confer resistance of pancreatic carcinoma cells against anticancer drug–induced cell death [23]. As far as apoptosis induced by single-agent treatment with different forms of Smac mimetics is concerned, NF-κB has been shown either to promote or to prevent cell death induction in different cancer cell lines. Whereas NF-κB inhibition was shown to potentiate Smac mimetic–triggered cytotoxicity in lung or prostate carcinoma cells [24,25], Vince et al. [9] reported that blocking NF-κB reduces Smac mimetic–mediated cell death in ovarian carcinoma and rhabdomyosarcoma cells. These findings point to a context-dependent role of NF-κB in Smac mimetic–induced cell death.

Furthermore, our study is the first to demonstrate that NF-κB is required for cell death induction after treatment with the combination of BV6 and gemcitabine. By comparison, TNFα has been implicated to mediate cell death induced by a Smac mimetic in combination with chemotherapeutics [26,27]. Our findings underscore that NF-κB may exert a tumor suppressor function under certain circumstances and raise a cautious note on the use of NF-κB inhibitors in pancreatic cancers in the context of Smac mimetics.

Our results have important implications for the future development of Smac mimetic–based combination protocols for the treatment of pancreatic cancer. Since the addition of BV6 markedly potentiates the antitumor activity of several cytotoxic drugs against pancreatic carcinoma cells, the incorporation of Smac mimetic into chemotherapy protocols can be considered as a promising strategy to be further exploited in the future. As Smac mimetics have recently entered phase 1 clinical trials [28], it is a timely question to develop combination protocols with these compounds to exploit synergistic drug actions. To this end, combinations with conventional chemotherapeutic agents offer the advantage that they might rapidly be transferred into clinical practice. In conclusion, the combination of the Smac mimetic BV6 with gemcitabine presents a promising strategy for apoptosis-targeted therapies of pancreatic cancer, which warrants further investigation.

Acknowledgments

The authors thank C. Hugenberg for expert secretarial assistance.

References

[1] Li D, Xie K, Wolff R, and Abbruzzese JL (2004). Pancreatic cancer. *Lancet* **363**, 1049–1057.

[2] Fulda S (2009). Apoptosis pathways and their therapeutic exploitation in pancreatic cancer. *J Cell Mol Med* **13**, 1221–1227.

[3] Fulda S and Debatin KM (2006). Extrinsic *versus* intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene* **25**, 4798–4811.

[4] Ashkenazi A (2008). Targeting the extrinsic apoptosis pathway in cancer. *Cytokine Growth Factor Rev* **19**, 325–331.

[5] Kroemer G, Galluzzi L, and Brenner C (2007). Mitochondrial membrane permeabilization in cell death. *Physiol Rev* **87**, 99–163.

[6] LaCasse EC, Mahoney DJ, Cheung HH, Plenchette S, Baird S, and Korneluk RG (2008). IAP-targeted therapies for cancer. *Oncogene* **27**, 6252–6275.

[7] Bertrand MJ, Milutinovic S, Dickson KM, Ho WC, Boudreault A, Durkin J, Gillard JW, Jaquith JB, Morris SJ, and Barker PA (2008). cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination. *Mol Cell* **30**, 689–700.

[8] Varfolomeev E, Goncharov T, Fedorova AV, Dynek JN, Zobel K, Deshayes K, Fairbrother WJ, and Vucic D (2008). c-IAP1 and c-IAP2 are critical mediators of tumor necrosis factor α (TNFα)-induced NF-κB activation. *J Biol Chem* **283**, 24295–24299.

[9] Vince JE, Wong WW, Khan N, Feltham R, Chau D, Ahmed AU, Benetatos CA, Chunduru SK, Condon SM, McKinlay M, et al. (2007). IAP antagonists target cIAP1 to induce TNFα-dependent apoptosis. *Cell* **131**, 682–693.

[10] Varfolomeev E, Blankenship JW, Wayson SM, Fedorova AV, Kayagaki N, Garg P, Zobel K, Dynek JN, Elliott LO, Wallweber HJ, et al. (2007). IAP antagonists induce autoubiquitination of c-IAPs, NF-κB activation, and TNFα-dependent apoptosis. *Cell* **131**, 669–681.

[11] Petersen SL, Wang L, Yalcin-Chin A, Li L, Peyton M, Minna J, Harran P, and Wang X (2007). Autocrine TNFα signaling renders human cancer cells susceptible to Smac-mimetic–induced apoptosis. *Cancer Cell* **12**, 445–456.

[12] Baud V and Karin M (2009). Is NF-κB a good target for cancer therapy? Hopes and pitfalls. *Nat Rev Drug Discov* **8**, 33–40.

[13] Vogler M, Walczak H, Stadel D, Haas TL, Genze F, Jovanovic M, Bhanot U, Hasel C, Moller P, Gschwend JE, et al. (2009). Small molecule XIAP inhibitors enhance TRAIL-induced apoptosis and antitumor activity in preclinical models of pancreatic carcinoma. *Cancer Res* **69**, 2425–2434.

[14] Vogler M, Walczak H, Stadel D, Haas TL, Genze F, Jovanovic M, Gschwend JE, Simmet T, Debatin KM, and Fulda S (2008). Targeting XIAP bypasses Bcl-2–mediated resistance to TRAIL and cooperates with TRAIL to suppress pancreatic cancer growth *in vitro* and *in vivo*. *Cancer Res* **68**, 7956–7965.

[15] Vogler M, Durr K, Jovanovic M, Debatin KM, and Fulda S (2007). Regulation of TRAIL-induced apoptosis by XIAP in pancreatic carcinoma cells. *Oncogene* **26**, 248–257.

[16] Fulda S, Friesen C, Los M, Scaffidi C, Mier W, Benedict M, Nunez G, Krammer PH, Peter ME, and Debatin KM (1997). Betulinic acid triggers CD95 (APO-1/Fas)- and p53-independent apoptosis via activation of caspases in neuroectodermal tumors. *Cancer Res* **57**, 4956–4964.

[17] Karl S, Pritschow Y, Volcic M, Hacker S, Baumann B, Wiesmuller L, Debatin KM, and Fulda S (2009). Identification of a novel pro-apopotic function of NF-κB in the DNA damage response. *J Cell Mol Med* **13**, 4239–4256.

[18] Kasperczyk H, La Ferla-Bruhl K, Westhoff MA, Behrend L, Zwacka RM, Debatin K-M, and Fulda S (2005). Betulinic acid as new activator of NF-κB: molecular mechanisms and implications for cancer therapy. *Oncogene* **24**, 6945–6956.

[19] Wang L, Du F, and Wang X (2008). TNF-α induces two distinct caspase-8 activation pathways. *Cell* **133**, 693–703.

[20] Degterev A, Huang Z, Boyce M, Li Y, Jagtap P, Mizushima N, Cuny GD, Mitchison TJ, Moskowitz MA, and Yuan J (2005). Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. *Nat Chem Biol* **1**, 112–119.

[21] Stadel D, Mohr A, Ref C, MacFarlane M, Zhou S, Humphreys R, Bachem M, Cohen G, Moller P, Zwacka RM, et al. (2010). TRAIL-induced apoptosis is

preferentially mediated via TRAIL receptor 1 in pancreatic carcinoma cells and profoundly enhanced by XIAP inhibitors. *Clin Cancer Res* **16**, 5734–5749.

[22] Greten FR, Weber CK, Greten TF, Schneider G, Wagner M, Adler G, and Schmid RM (2002). Stat3 and NF-κB activation prevents apoptosis in pancreatic carcinogenesis. *Gastroenterology* **123**, 2052–2063.

[23] Arlt A, Gehrz A, Muerkoster S, Vorndamm J, Kruse ML, Folsch UR, and Schafer H (2003). Role of NF-κB and Akt/PI3K in the resistance of pancreatic carcinoma cell lines against gemcitabine-induced cell death. *Oncogene* **22**, 3243–3251.

[24] Bai L, Chen W, Wang X, Tang H, and Lin Y (2009). IKKβ-mediated nuclear factor-κB activation attenuates Smac mimetic–induced apoptosis in cancer cells. *Mol Cancer Ther* **8**, 1636–1645.

[25] Petersen AM, Jung WS, Yang JS, and Stanley HE (2011). Quantitative and empirical demonstration of the Matthew effect in a study of career longevity. *Proc Natl Acad Sci USA* **108**, 18–23.

[26] Dineen SP, Roland CL, Greer R, Carbon JG, Toombs JE, Gupta P, Bardeesy N, Sun H, Williams N, Minna JD, et al. (2010). Smac mimetic increases chemotherapy response and improves survival in mice with pancreatic cancer. *Cancer Res* **70**, 2852–2861.

[27] Probst BL, Liu L, Ramesh V, Li L, Sun H, Minna JD, and Wang L (2010). Smac mimetics increase cancer cell response to chemotherapeutics in a TNF-α–dependent manner. *Cell Death Differ* **17**, 1645–1654.

[28] Fulda S (2009). Inhibitor of apoptosis proteins in hematological malignancies. *Leukemia* **23**, 467–476.

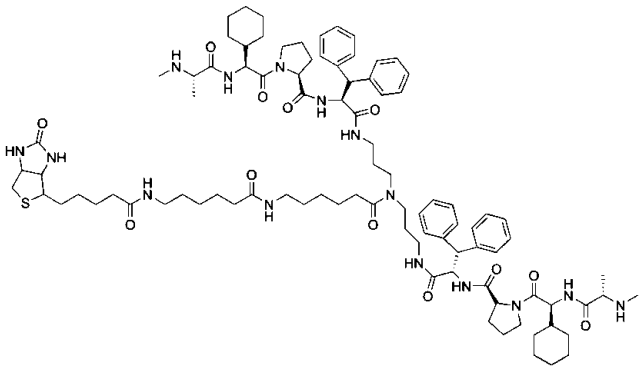

Figure W1. Structure of BV6. The structure of BV6 is shown according to Varfolomeev et al. [10].

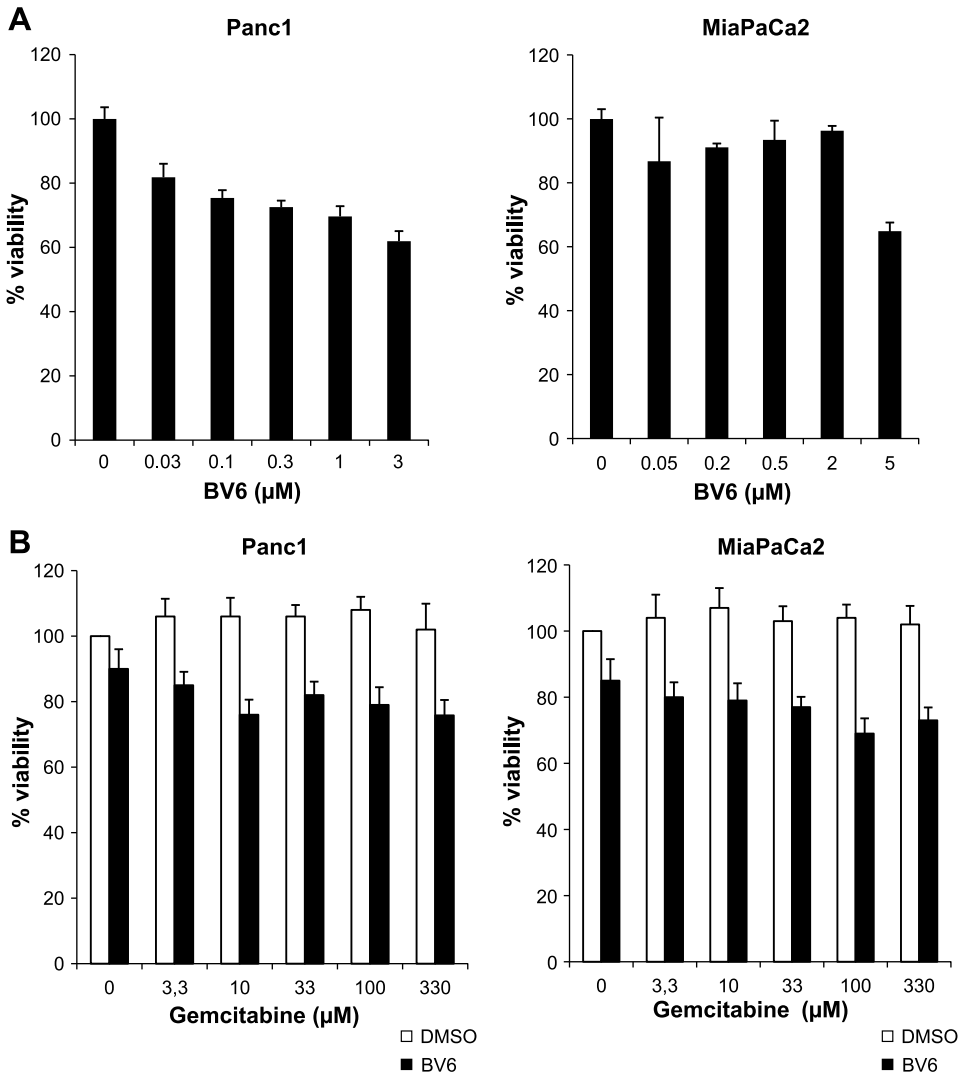

Figure W2. Dose-response and kinetic analysis. Panc1 (left panels) and MiaPaCa2 (right panels) cells were treated with indicated concentrations of BV6 for 72 hours (A) or with indicated concentrations of gemcitabine in the presence (black bars) or absence (white bars) of 2 μM BV6 for 24 hours (B). Cell viability was assessed by MTT assay and is calculated as percentage of untreated cells. Means ± SEM of three independent experiments performed in triplicate are shown.

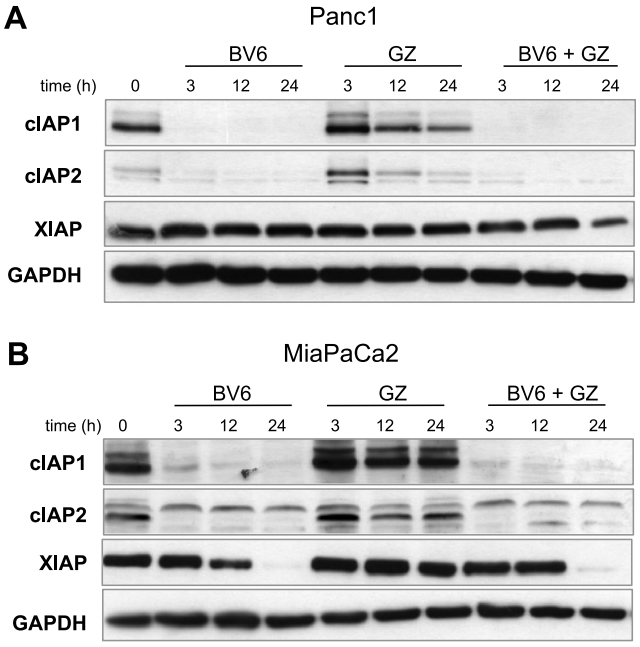

Figure W3. Effect of BV6 on expression of IAP proteins. Panc1 (upper panel) and MiaPaCa2 (lower panel) cells were treated with 100 nM (Panc1) or 66 nM (MiaPaCa2) gemcitabine and/or 2 μM BV6 for indicated time points. The expression of cIAP1, cIAP2, and XIAP was assessed by Western blot analysis. GAPDH served as loading control. One representative Western blot of two independent experiments is shown.

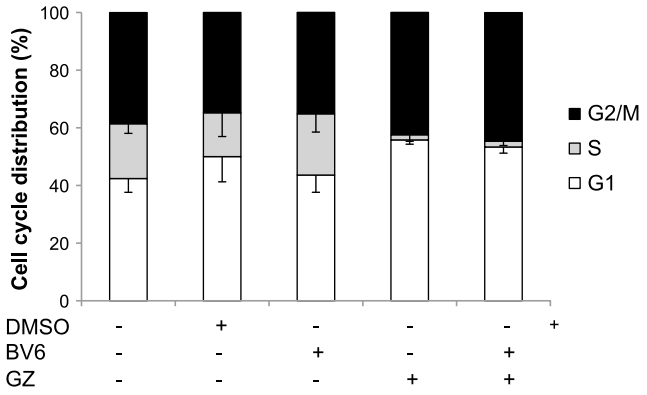

Figure W4. Effect of BV6 on the cell cycle. Panc1 cells were treated with 2 μM BV6 and/or 100 nM gemcitabine for 72 hours. Cell cycle profiles were assessed by propidium iodide staining of permeabilized cells and flow cytometry gating on alive cells. Percentages of cells in G_1, G_2/M, and S phases of the cell cycle are shown as mean ± SEM of three independent experiments.

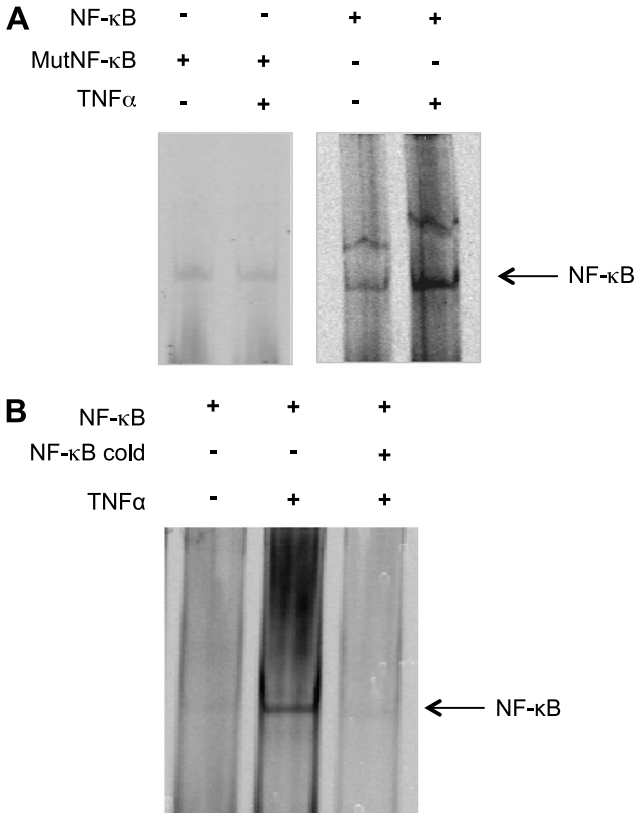

Figure W5. BV6- and gemcitabine-induced NF-κB activation. Panc1 cells were treated for 1 hour with 10 ng/ml TNFα. NF-κB activation was determined by EMSA. (A) Nuclear protein extracts prepared from unstimulated or TNFα-stimulated cells were incubated with labeled NF-κB oligonucleotides presenting consensus NF-κB sequence (NF-κB) or mutated NF-κB sequence (MutNF-κB). (B) Cold competition of EMSA assay was performed using an excess (250 fmol) of unlabeled NF-κB oligonucleotides (NF-κB cold).

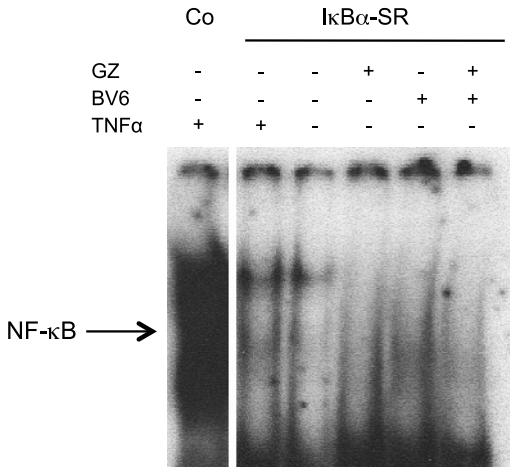

Figure W6. Effect of IκBα-SR on BV6- and gemcitabine-induced NF-κB activation. Panc1 cells stably transduced with a vector containing IκBα-SR (IκBα-SR) or empty vector control (Co) were treated for 4 hours with 2 μM BV6 and/or 100 nM gemcitabine or for 1 hour with 10 ng/ml TNFα. NF-κB activation was determined by EMSA.