Aus dem Fachbereich Medizin

der Johann Wolfgang Goethe-Universität

Frankfurt am Main

betreut am

Zentrum der Kinder- und Jugendmedizin

Institut für Experimentelle Tumorforschung in der Pädiatrie

Direktorin: Prof. Dr. med. Simone Fulda

Evaluation of the bivalent Smac mimetic BV6 and Interferon α as a combination treatment in Glioblastoma multiforme

Dissertation

zur Erlangung des Doktorgrades der Medizin

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Meinen Eltern

TABLE OF CONTENTS

Table of contents 4
Acknowledgements 8
Abstract 9
Zusammenfassung 10
List of abbreviations 12
Table of figures 15
Table of tables 16
1 Introduction 17
1.1 Glioblastoma multiforme 17
1.1.1 Overview and epidemiology 17
1.1.2 Tumorigenesis of human GBM 18
1.2 Signaling pathways of apoptosis 19
1.2.1 Overview 19
1.2.2 Caspases as mediators of cellular apoptosis 20
1.2.3 The extrinsic pathway 21
1.2.4 The intrinsic pathway 23
1.3 Inhibitors of apoptosis 26
1.3.1 The IAP-family 26
1.3.2 Molecular structure of IAPs 27
1.4 Smac mimetics 29
1.4.1 Structure of Smac mimetics 29
1.4.2 Inhibition of IAPs 31
1.4.3 Smac mimetic-based combination protocols 33
1.4.4 Smac mimetics in clinical trials 33
1.5 NFκB 36
1.5.1 The NFκB family 36

1.5.2 The canonical NFκB pathway 36
1.5.3 The non-canonical NFκB pathway 38
1.5.4 NFκB in tumorigenesis 39
1.6 Interferon α 42
1.6.1 Taxonomy and biological properties of Interferons 42
1.7 Aims of the study 46
2 Material and methods 48
2.1 Material 48
2.1.1 Cell lines 48
2.1.2 Cell culture and cultivation 49
2.1.3 Cell death induction and inhibition 49
2.1.4 General chemicals 49
2.1.5 Electrophoresis and Western Blotting 50
2.1.6 Buffers and solutions 51
2.1.7 Antibodies 53
2.1.8 Plastic materials 54
2.1.9 Kits 55
2.1.10 Equipment and tools 55
2.2. Cell culture 56
2.2.1 Cell cultivation and splitting 56
2.2.2 Cell seeding and treatment 57
2.3 Cell death assays 57
2.3.1 Determination of cell viability via MTT assay 57
2.3.2 Determination of DNA fragmentation by flow cytometry 58
2.3.3 Determination of membrane permeabilization by flow cytometry 59
2.3.4 NFκB reporter assay 59
2.4 Protein analysis and statistical methods 60
2.4.1 Cell lysis and protein extraction 60

2.4.2 SDS polyacrylamide gel electrophoresis 60
2.4.3 Western blot 61
2.4.4 Statistical analysis 61
3 Results 63
3.1 BV6 and IFNα as single treatment 63
3.1.1 BV6 induces cell death in GBM cells in a dose-dependent manner 63
3.1.2 IFNα single treatment reduces cell viability in GBM cells in a dose-dependent manner 66
3.1.3 BV6 and IFNα induce DNA fragmentation in GBM cell lines 68
3.2 BV6 and IFNα as combination treatment 70
3.2.1 BV6 and IFNα cooperate to induce loss of cell viability in GBM cells 70
3.2.2 BV6 and IFNα cooperate to induce DNA fragmentation in GBM cells 71
3.2.3 BV6 and IFNα cooperate to induce membrane permeabilization in GBM cells 73
3.2.4 Synergistic interaction of BV6 and IFNα is confirmed by CI calculation 75
3.3 Molecular mechanism of cell death induction by BV6/IFNα 76
3.3.1 Inhibition of caspases effectively impairs induction of cell death by BV6/IFNα-cotreatment 76
3.3.2 BV6/IFNα-cotreatment induces activation of NFκB signaling 79
3.3.3 Inhibition of NFκB activation rescues cells from BV6/IFNα-induced cell death 81
3.3.4 BV6/IFNα-cotreatment induces expression of NFκB-responsive genes 85
3.3.5 BV6/IFNα-cotreatment induces cell death independent of extracellular TNFα or TRAIL 87
3.4 Concluding remarks 88
4 Discussion 89
4.1 BV6 and IFNα cooperate to induce cell death in GBM cells in a synergistic manner 89
4.2 The induction of cell death by BV6/IFNα-cotreatment depends on NFκB signaling and caspase activation. 90

4.3 Induction of cell death by BV6/IFNα is independent of TNFα and soluble TRAIL 91

4.4 Additional NFκB target genes might be involved in BV6/IFNα-induced cell death 93

4.5 Summary and further outlook 94

References 96

Schriftliche Erklärung 132

Lebenslauf 134

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ABSTRACT

Glioblastoma multiforme accounts for more than 80% of all malignant gliomas in adults and a minor fraction of new annual cases occurs in children. In the last decades, research shed light onto the molecular patterns underlying human malignancies which resulted in a better understanding of the disease and finally an improved long term survival for cancer patients. However, malignancies of the central nervous system and especially glioblastomas are still related to poor outcomes with median survivals of less than 6 months despite extensive surgery, chemotherapy and radiation. Hence, a better understanding of the molecular mechanism driving and sustaining cancerous mutations in glioblastomas is crucial for the development of targeted therapies. Apoptosis, a form of programmed cell death, is an important feature of eukaryotic cells and crucial for the maintenance of multicellular homeostasis. Because apoptosis is a highly complex and tightly regulated signaling pathway, resisting apoptotic stimuli and avoiding cell death is a hallmark of the cancerous transformation of cells. Hence, targeting molecular structures to reestablish apoptotic signaling in tumor cells is a promising approach for the treatment of malignancies. Smac mimetics are a group of small molecular protein inhibitors that structurally derive from an intracellular protein termed Smac and selectively block Inhibitor of apoptosis (IAP) proteins, which are often aberrantly expressed in cancer. Several studies confirmed the antitumoral effects of Smac mimetics in different human malignancies, including glioblastoma, and give rationales for the development of potent Smac mimetics and Smac mimetic-based combination protocols. This study investigates the antitumoral activity of the bivalent Smac mimetic BV6 in combination with Interferon α. Latter is a well characterized cytokine with an essential role in immunity, cell differentiation and apoptosis. This study further aims to address the molecular mechanisms underlying the antitumoral activity of the combination treatment by using well established molecular cell death assays, flow cytometry, western blot analysis, genetic approaches and selective pharmacological inhibition. Since different Smac mimetics and Smac mimetic-based combination therapies are currently under clinical evaluations, findings of this study may have broad implications for the application of Smac mimetics as clinical cancer therapeutics.

ZUSAMMENFASSUNG

Glioblastoma multiforme stellt mit etwa 80% den größten Anteil der malignen Hirntumoren bei erwachsenen Patienten dar. Ein kleiner Teil der jährlichen Neudiagnosen des Glioblastoms entfällt dabei auf Kinder. Zwar konnten in den vergangenen Jahrzenten wichtige Einblicke in die Zugrunde liegenden molekularen Veränderungen von Krebserkrankungen gewonnen werden, dennoch haben Malignitäten des zentralen Nervensystems und besonders das Glioblastom noch immer eine sehr schlechte Prognose mit einer mittleren Überlebenszeit von weniger als 6 Monaten. Daher ist ein besseres Verständnis der molekularen Veränderungen, welche die maligne Transformation in Glioblastomen befördern und unterhalten, von essentieller Bedeutung für die Entwicklung von modernen Krebstherapien. Apoptose, eine Form des programmierten Zelltodes, ist ein wichtiger Mechanismus in eukaryotischen Zellen und grundlegend für die Regulation und den Erhalt von multizellularer Homöostase. Aufgrund der enormen Komplexität und der strengen Regulation von Apoptose wenden Krebszellen unterschiedliche Strategien an, um apoptotischen Stimuli und damit dem Zelltod zu entgehen. Das gezielte Unterbinden dieser Mechanismen und die Resensitivierung der Krebszellen auf apoptotische Stimuli ist daher ein vielversprechender therapeutischer Ansatz. Smac Mimetika sind eine Gruppe von niedermolekularen Proteininhibitoren welche mit dem Ziel entwickelt wurden, Apoptose-Inhibitoren (engl. Inhibitor of apoptosis (IAP)), eine in Tumorzellen häufig fehlregulierte Proteinfamilie, zu blockieren. Die antitumorale Wirkung solcher Smac Mimetika konnte im Rahmen zahlreicher Studien in unterschiedlichen Tumorentitäten, darunter dem Glioblastom, gezeigt werden. Diese Studien liefern eine Rationale für die Weiterentwicklung dieser niedermolekularen Proteininhibitoren sowie die Erprobung dieser Therapeutika in unterschiedlichen Kombinationstherapien. Die vorliegende Studie untersucht im Rahmen eines solchen Kombinationsprotokolls die antitumorale Wirkung des bivalenten Smac Mimetikums BV6 mit Interferon α, einem humanen Zyotkin, welches eine wichtige Rolle in der angeborenen Immunität, der Zelldifferenzierung und der Apoptoseregulation spielt. Die vorliegende Studie verwendet dabei etablierte molekularbiologische Methoden, etwa unterschiedliche Zelltod-Assays, Durchflusszytometrie, Western Blot-Analysen, gentechnische Methoden sowie gezielte pharmakologische Inhibitionen, um die grundlegenden molekularen Mechanismen dieser Kombinationsbehandlung zu identifizieren. Zum aktuellen Zeitpunkt werden verschiedene Smac Mimetika in klinischen Studien auf ihren

therapeutischen Nutzen getestet, so dass die in der vorliegenden Studie gewonnenen Erkenntnisse einen Zugewinn für die Entwicklung und Anwendungen von Smac Mimetika zur klinischen Tumortherapie sein können.

LIST OF ABBREVIATIONS

ATCC	American type culture collection
Apaf1	Apoptotic protease activating factor 1
AVPI	Ala-Val-Pro-Ile
BAFF-R	B cell activating factor receptor
Bcl-2	B cell lymphoma-2
BID	BH3-interacting domain death agonist
BIRC1	Neuronal apoptosis inhibiting protein
BIRC5	Survivin
BIRC6	Ubiquitin-conjugated BIR domain enzyme apollon
CARD	Caspase recruitment domain
CDK	Cyclin-dependent kinase
CI	Combination Index
cIAP1	Cellular inhibitor of apoptosis 1
cIAP2	Cellular inhibitor of apoptosis 2
CNS	Central nervous system
DD	Death domain
DISC	Death inducing signaling complex
DR	Death receptor
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin E3-ligase
EGFR	Epidermal growth factor receptor
FADD	Fas-associated protein with death domain
FasL/CD95	Fas ligand

FCS	Fetal calf serum
GBM	Glioblastoma multiforme
HRP	Horseradish peroxidase
IBM	IAP-binding motif
IFNAR	Interferon type 1/α receptor
IKK	IκB-kinase
Il-1R	Interleukin-1 receptor
ILP2/BIRC8	IAP-linked protein 2
Interferon	IFN
IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene
ISGF	Interferon-stimulated gene factor
ISRE	Interferon-stimulated response element
IκBα	Inhibitor of κBα
JAK	Janus activated kinase
LTβR	Lymphotoxin β receptor
ML-IAP/BIRC7	Melanoma IAP
MMP	Mitochondrial membrane permeabilization
MTS	Mitochondrial targeting sequence
NEMO	NFκB essential modulator
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIK	NFκB inducing kinase
PTEN	Phosphatase and tensin homolog
RANK	Receptor activator of NFκB

RHD	Rel homology domain
RING	Really interesting new gene
RIPK1/3	Receptor-interacting serine/threonine kinase 1/3
ROS	Reactive oxygen species
SH2	Src homology 2
Smac	Second mitochondria derived factor of caspase
STAT	Signal transducer and activator of transcription
TF	Transcription factor
TIR	TLR and IL-1R homology domain
TLR	Toll-like receptor
TNFR1	TNF receptor 1
TNFα	Tumor necrosis factor α
TRADD	TNF receptor-associated death domain protein
TRAF	TNF receptor associated factor
TRAIL/Apo2L	TNF-related apoptosis inducing ligand
TRAILR 1/2	TRAIL receptor 1 and 2
Ub	Ubiquitin
xIAP/BIRC4	X-linked inhibitor of apoptosis
Δψm	Mitochondrial intermembrane potential

TABLE OF FIGURES

Figure 1 Overview of the apoptotic pathway 25
Figure 2 Schematic representation of the seven human IAP family members 28
Figure 3 Structure of Smac and N-terminal AVPI-motif. 31
Figure 4 Overview of the cellular effects of Smac mimetics. 35
Figure 5 Overview of the NFκB signaling pathway. 40
Figure 6 Overview of the IFNα signaling pathway 45
Figure 7 BV6 induces loss of cell viability in a dose-dependent manner. 64
Figure 8 IFNα induces loss of cell viability in a dose-dependent manner. 67
Figure 9 BV6 induces DNA fragmentation in a dose-dependent manner 68
Figure 10 IFNα induces DNA fragmentation in a dose-dependent manner. 69
Figure 11 The combination of BV6 and IFNα induces loss of cell viability in a dose-dependent manner 70
Figure 12 The combination of BV6 and IFNα induces DNA fragmentation in Glioblastoma cells in a dose-dependent manner 72
Figure 13 The combination of BV6 and IFNα induces permeabilization of the cell membrane in Glioblastoma cells in a dose-dependent manner. 74
Figure 14 Caspases play an important role in the induction of loss of cell viability and DNA fragmentation induced by the combination of BV6 and IFNα 77
Figure 15 BV6 and IFNα cooperate to trigger depletion of IAP proteins and activate key proteins of the NFκB pathway in A172 and U87 Glioblastoma cells 80
Figure 16 In A172 and U87 IκBα-SR cells, TNFα fails to induce phosphorylation of IκBα and subsequent activation of the NFκB signaling pathway 82
Figure 17 Inhibition of the canonical NFκB pathway diminishes the loss of cell viability and the DNA fragmentation that is induced by the combination of BV6 and IFNα 84
Figure 18 NFκB activity is increased after treatment with BV6 and the combination of BV6 and IFNα 85
Figure 19 BV6 and IFNα corporately trigger loss of cell viability and DNA fragmentation in Glioblastoma cells mainly independent of TNFα and TRAIL 87

TABLE OF TABLES

Table 1 CI calculation for the BV6/IFNα cotreatment in A172 and U87 based on the results of DNA fragmentation. 75

1 INTRODUCTION

1.1 Glioblastoma multiforme

1.1.1 Overview and epidemiology

Since the U.S. declared the war against cancer in 1971, the world wide efforts to find novel ways of preventing and treating cancer grew rapidly within the last four decades. Extensive research shed light onto the complex biology of tumor cells and the molecular patterns that underlie the malignant transformation, which were used to improve treatment protocols. These efforts resulted in better diagnostics, therapies and long term survival for patients with cancer. This is one reason why the rate of cancer related mortality declines, even though the incidence of malignant diseases keeps growing.

While some of the patients benefit from these improvements, for example patients with malignant tumors of the colon, the stomach or the prostate, there are still cancer entities related to a poor prognosis, including malignancies of the central nervous system (CNS). One of the most common and aggressive primary malignancies of the CNS is the glioblastoma multiforme (GBM).

GBM accounts for over 80% of all malignant gliomas.[1] It has a peak occurrence in adults older than 50 years and less than 10% of all GBM occur in children. In children, tumors of the CNS are the most common solid tumors, though GBM are responsible for a minor fraction of 0.14 new annual cases per 100.000 children.[2]

Originally, astrocytes were thought to be the cells of origin of GBMs.[3] However, recent research points out that there are various types of less-differentiated, stem-cell like populations within the tumor.[4]

As for the most CNS tumors there are just a few factors known to play a causal role in the tumorigenesis of GBMs. Unlike most tumor entities, the incidence of CNS tumors shows no correlation with environmental factors like nutrition or smoking and there is

no verified evidence that toxins or pathogens like bacteria or viruses have an influence on the tumorigenic process.[5] Furthermore, it could not be shown that people exposed to electromagnetic radiation carry a higher risk to develop GBM. There is an increased risk for GBM in people that underwent therapeutic radiation of the head during their youth but on the contrary this correlation cannot be seen for ionizing radiation that is used for imaging techniques.[5]

A family history of malignant brain tumors is another established risk factor for brain cancer. Children of parents with malignant CNS tumors have a higher risk to develop CNS tumors themselves. The same holds true for siblings of CNS tumor patients.[2] These epidemiological findings suggest that hereditary genetic alterations can predispose for the development of malignant brain tumors.

1.1.2 Tumorigenesis of human GBM

GBMs can be divided into primary GBMs, which develop *de novo* due to accumulation of oncogenic mutations and secondary GBMs that arise from WHO high grade II and III astrocytomas.[6] Current research suggests that both subtypes differ genetically but not histologically nor in malignancy.[7] The majority of primary GBMs shows overexpression of the epidermal growth factor receptor (EGFR) but rarely mutations of the tumor suppressor p53. In contrast, secondary GBMs have a high incidence of p53 mutations but only minor EGFR overexpression.[8] Additional mutations that are common in both forms of GBMs are a partial or complete loss of chromosome 10 (10p and 10q).[7,9,10] Chromosome 10 carries the gene for the phosphatase and tensin homolog (PTEN)-phosphatase that is frequently deleted in many human cancers and is known to play an important role in maintaining chromosomal stability.[7,11,12] These mutations are linked to characteristics of malignant brain tumors such as hyperproliferation, hypervascularisation and resistance to apoptosis.[7] Thus, genomic instability and subsequent mutations endow cells with genetic alterations that drive tumor progression in GBM.[13]

1 Introduction

Apoptosis is a form of programmed cell death and has been intensively studied for decades. Since it serves as a natural barrier against cancer development, the ability to evade apoptosis is one of the hallmarks of cancer cells.[13] Apoptosis acts as a counterbalance to cell proliferation and growth and as such maintains tissue homeostasis. Resistance against apoptotic signals thus prevents effective elimination of cancer cells. Furthermore, most cancer therapies, including chemotherapy, radiation and immunotherapy act by inducing apoptosis in cancer cells. Evading apoptosis does not only promote cancer development, but can confer resistance against widely used therapeutic regimens.[14–16]

1.2 Signaling pathways of apoptosis

1.2.1 Overview

During their lifespan, eukaryotic cells are constantly subjected to a vast amount of potentially lethal stimuli. These stimuli can trigger a number of different pathways that evolved in order to dispose cells in a regulated manner. Amongst them, apoptosis is the best characterized and most conserved form of cell death. But other forms of regulated cell death have gained recent interest in the research community. In general, the pathways can be subdivided in apoptosis, autophagy, necroptosis and others (such as necrosis, ferroptosis or pyroptosis).[17,18] It is widely accepted that the most important role for apoptosis is the maintenance of multicellular homeostasis. This is supported by numerous reports which show that dysregulation in apoptotic cell death contributes to a large number of diseases.[19,20] In principal, apoptosis can be subdivided into extrinsic apoptosis which is induced by extracellular stress signals and intrinsic, mitochondria-dependent apoptosis.[21] Both eventually result in the activation of death effector molecules termed caspases.[22] Caspase activation is followed by the degradation of cellular structures. Phenotypic characteristics of cells undergoing apoptosis are the condensation and degradation of the DNA, fragmentation of vital organelles like the

endoplasmic reticulum as well as the Golgi apparatus and the constriction of the cellular membrane which results in membrane buds known as apoptotic bodies.[23]

Apoptotic cells express molecular markers on their outer surface as membrane asymmetry is lost. Eventually, phosphatidylserine, a phospholipid, is flipped to the extracellular side of the lipid bilayer.[24–26] Externalized phosphatidylserine and other lipoproteins serve as chemotactic stimuli for phagocytes such as macrophages that migrate towards the cells fragments and remove them rapidly and efficiently.[27,28] During the process of apoptosis, the cells break down into apoptotic bodies that surround intracellular structures. These bodies are engulfed by phagocytosis, which avoids the release of cellular debris that would otherwise elicit inflammation and perturb the surrounding tissue.[29]

1.2.2 Caspases as mediators of cellular apoptosis

Caspases are evolutionarily conserved cysteine-dependent aspartate-specific endoproteases. They are considered to represent the main executers of apoptosis.[30,31] Seven of the fifteen caspases expressed in mammalian cells are known to play a role in apoptosis while others mediate proteolytic activation of inflammatory cytokines.[32] The seven apoptotic caspases can be further subdivided into initiator caspases (caspase-2, caspase-8, caspase-9 and caspase-10) and effector caspases (caspase-3, -6 and -7). Caspases are primarily produced as catalytically inactive zymogens and gain proteolytic activity following signaling events.[33] They consist of three parts, namely the NH_2-terminus prodomain, called death effector domain (DED or CARD), a large domain (p20) containing the active catalytic subunit and a small domain (p10), also called small catalytic subunit. Activated caspases can hydrolyze peptide bonds on the carboxy-terminal side of an aspartate-residue. During the process of activation, initiator caspases like caspase-8, are recruited to molecular platforms, such as DISC and complex II and are cleaved twice at specific aspartate-residues. The generated subunits can form catalytically active heterotetramers to initiate apoptosis. This allows activated caspases

to efficiently process and activate other zymogens. After the cleavage at the DISC, the mature caspase-8 is released into the cytosol where it activates additional caspases and triggers apoptosis. The tightly regulated process of cleavage-dependent activation of caspase-8 most likely avoids unintentional activation of the apoptotic pathway.

1.2.3 The extrinsic pathway

The extrinsic pathway is initiated by the binding of death ligands to their corresponding extracellular death receptors (DR 1-6).[34] All ligands belong to the Tumor necrosis factor (TNF) superfamily. This family comprises Tumor necrosis factor α (TNFα), TNF-related apoptosis inducing ligand (TRAIL/Apo2L), Fas ligand (FasL/CD95L) and additional cytokines.[35] The best characterized death receptors are CD95/Fas, TNF receptor 1 (TNFR1/DR1) and TRAIL receptor 1 and 2 (TRAILR1 and TRAILR2).[21,36–40] TNFR1 is an ubiquitously expressed type I trans-membrane protein that resides in the cellular membrane. Binding of TNFα results in trimerization of the receptor units, providing an intracellular scaffold for signaling units.[41] The death domain (DD) of TNFR1 can associate with the TNF receptor-associated death domain protein (TRADD) and further recruit TNF receptor-associated factor 2 (TRAF 2), receptor-interacting serine/threonine kinase 1 (RIPK1) and the cellular inhibitor of apoptosis 1 and 2 (cIAP 1, 2), forming complex I.[42–44] The assembly of this complex takes place close to the cellular membrane and aids the stimulation of two key signaling pathways. First, complex I stimulates the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB)-pathway, a signaling pathway involved in cell survival and inflammation.[45,46] Second, it activates mitogen-activated protein kinase/c-Jun N-terminal kinase (MAPK/JNK) which results in the phosphorylation of the c-Jun transcription factor involved in survival signaling.[34] The process is regulated amongst others by non-degradative polyubiquitination of RIPK1, a post-translational modification in which ubiquitin chains are attached to specific lysine residues on the substrate.[47–49] In the case of complex I, the ubiquitination is induced by cIAP1 that acts as an E3 ubiquitin ligase.[47] Hence, constitutive ubiquitination of RIPK1 by cIAP 1 and cIAP

2 is considered to be a key regulatory process that elicits an anti-apoptotic response following TNFR1 activation.[50,51]

In contrast, deubiquitination of RIPK1 disassembles complex I and facilitates the formation of one of two cytosolic complexes.[52] Cytosolic TRADD oligomerizes and recruits Fas-associated protein with death domain (FADD), RIPK1 and procaspase-8 to form complex IIA.[53] Alternatively, cytosolic RIPK1 interacts with RIPK3 forming complex IIB/Necrosome which initiates an additional form of regulated cell death, termed necroptosis.[42,43] Assembled complex IIA cleaves procaspase-8, the main initiator caspase of extrinsic apoptosis, to its active cleavage fragments.[41,54] Complex IIB forms upon the inhibition of caspase activation, e.g. by pan-caspase inhibitor zVAD.fmk and induces necroptosis, an alternative form of programmed cell.[55,56]

Similar to TNFα, TRAIL induces apoptosis by binding to DR4 or DR5. Both receptors reside in the cellular membrane and binding of TRAIL leads to the formation of the death-inducing signaling complex (DISC), similar to complex IIA. This complex consists of the endogenous adapter molecule FADD and procaspase-8 and/or procaspase-10.[40,57] The binding of procaspase-8 and/or -10 to FADD results in its oligomerization and autoactivation.[58,59]

Similarly, binding of membrane-bound FasL (CD95L) to Fas (CD95/DR2) allows the formation of an intracellular death-inducing complex (DISC), consisting of FADD and procaspase-8 and/or procaspase-10.[36] Eventually, all death inducing ligands (TNFα, TRAIL and FasL) drive the activation of caspase-8 or caspase-10.[60–62]

Cells differ between two distinct signaling pathways following CD95-death-receptor induced apoptosis.[63–65] So called type I cells have high levels of DISC formation and increased amount of cleaved caspase-8 that can directly activate downstream effector caspase-3 and -7.[63] In contrast, type II cells only display minor DISC formation and low levels of activated caspase-8.[63,66] In these cells, downstream apoptotic signaling relies on the caspase-8-dependent cleavage of the pro-apoptotic Bcl2-family protein BH3-interacting domain death agonist (BID) to its truncated fragment (tBID) and subsequent induction of intrinsic apoptosis.[64]

1.2.4 The intrinsic pathway

The intrinsic or mitochondrial pathway of apoptosis is activated by endogenous stress signals such as withdrawal of growth factors, DNA damage and a large variety of other intracellular stimuli.[67,68] Induction of mitochondrial apoptosis depends on the balance of B cell lymphoma-2 (Bcl-2) family proteins, a divergent group of pro- and anti-apoptotic factors. They are divided according to the number and structure of their Bcl-2 homolog motif (BH 1-4).[69] One arm of the family, including Bcl-2, Bcl-X_L, Mcl-1 and others is necessary for cellular survival and inhibition of apoptosis.[69,70] On the contrary, BAX and BAK as well as BH3-only proteins such as BIM or PUMA induce apoptosis via the mitochondrial pathway.[71–73] However, the signaling cascades that induce mitochondrial apoptosis are very heterogeneous and remain an intense area of research. Eventually, all signaling pathways converge on the level of the mitochondrial membranes which are permeabilized when the lethal signals predominate over the survival signals.[21,67,74,75] Activated BAX and BAK oligomerizes and forms pores resulting in mitochondrial outer membrane permeabilization (MOMP).[76–78] In most cases, MOMP is the crucial event in the execution of mitochondrial apoptosis and a “point of no return”.[79,80] MOMP triggers the release of cytochrome c, second mitochondria derived factor of caspase (Smac), loss of the mitochondrial intermembrane potential ($\Delta\psi_m$) and the bioenergetic catastrophe including the arrest of the respiratory chain prior to the accumulation of reactive oxygen species (ROS).[75,81,82] Cytosolic cytochrome c promotes conformational changes of the apoptotic protease activating factor 1 (Apaf1).[83–85] Activated Apaf1 binds desoxyadenosine triphosphate (dATP) as a cofactor and oligomerizes forming a heptameric wheel-like structure called the Apoptosome.[86] This protein complex can recruit and activate the initiator caspase-9.[87–89]

The final cog in both apoptotic pathways is activation of the executioner caspase-3 and structurally similar caspase-7.[90] In the case of the intrinsic pathway, activated caspase-9 can rapidly cleave and activate the effector caspases. Similarly, caspase-8 takes up this task in type I cells, initiating the death cascade. Hence, effector caspase-3 and -7 are the

point of convergence for the extrinsic, death-receptor dependent pathway and the mitochondrial pathway of apoptotic cell death.[91]

Activated caspase-3 and caspase-7 can cleave several key substrates in the cells in order to activate enzymes necessary for the execution of apoptosis. One example is the liberation of the caspase dependent DNase (CAD) through cleavage of its inhibitor (ICAD), which mediates the apoptotic DNA fragmentation as a classical feature of this form of cell death.[92,93]

In general, apoptosis is a highly conserved, well understood and tightly regulated form of cell death. The extrinsic pathway is activated in response to binding of lethal ligands while activation of the intrinsic, mitochondrial pathway is the result of various endogenous stress signals. Both pathways eventually result in the activation of caspases, the main executors of apoptosis. Cells undergoing apoptosis are morphologically characterized by DNA fragmentation and formation of apoptotic bodies that carry intracellular structures. Mechanisms to avoid apoptosis are on the one hand crucial for the maintenance of tissue homeostasis. On the other hand, malignant cells can harbor these mechanisms in order to evade cell death.

1 Introduction

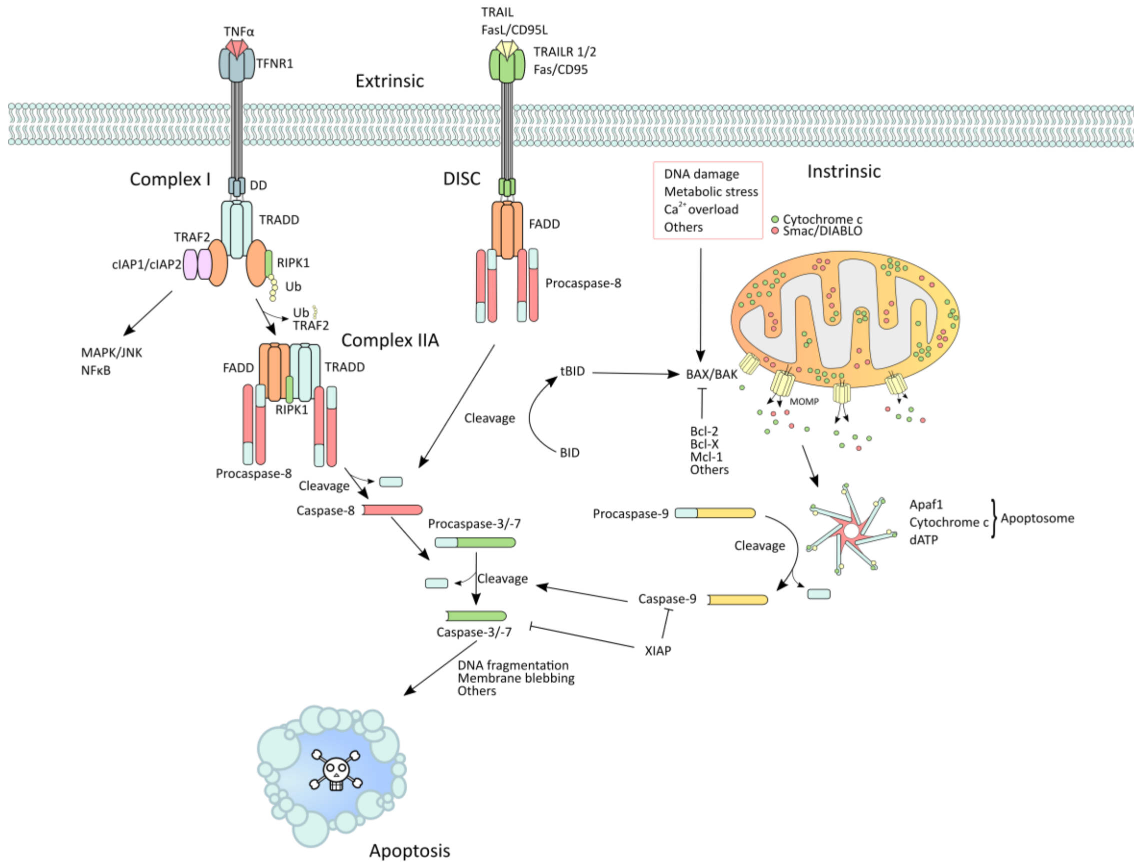

Figure 1 Overview of the apoptotic pathway

The extrinsic pathway of apoptosis is activated in response to the binding of lethal ligands (TNFα, TRAIL, FasL/CD95L) to their cognate cell-surface receptors (TNFR1, TRAILR 1/2, Fas/CD95). Binding of TNFα results in the trimerization of the receptor units, formation of the intracellular death domain (DD) and recruitment of TRADD, TRAF2, cIAP1/cIAP2 and RIPK1 into complex I. This aids the stimulation of two key signaling pathways, MAPK/JNK and NFκB. RIPK1 is eventually deubiquitinated and the subsequent disruption of complex I is followed by the liberation of TRADD into the cytosol. Monomeric TRADD recruits FADD and procaspase-8 to assemble complex IIA, which facilitates caspase-8 cleavage activating apoptosis via caspase-3 and caspase-7. Likewise, TRAIL and ApoL/CD95L induce extrinsic apoptosis by engaging TRAILR 1/2 and Apo, respectively, in order to induce intracellular DISC formation and caspase-8 activation along with BID truncation. The intrinsic/mitochondrial pathway is initiated by DNA damage, metabolic stress, Ca^{2}+ overload and other endogenous factors that converge on the level of BH3-only proteins altering mitochondrial membrane potential and permeability (MOMP). Release of cytochrome c along with Smac activates Apaf1 and induces dATP-dependent formation of the heptameric Apoptosome which leads to the activation of caspase-9 and later caspase-3 and caspase-7. Cells undergoing apoptosis are morphologically characterized by DNA fragmentation and blebbing of the cellular membrane into apoptotic bodies.

1.3 Inhibitors of apoptosis

1.3.1 The IAP-family

Inhibitors of apoptosis (IAP) are key regulator proteins in apoptotic cell death, innate immunity and immune response.[94] IAPs directly bind and inhibit caspases, the main executors of cellular apoptosis. This family of proteins is not exclusively found in eucaryotic cells but was first discovered in baculoviruses, where they block apoptosis in the infected host cells to enhance viral replication.[95] All members of the IAP family contain one to three baculovirus IAP repeats (BIR)-domains (BIR1-BIR3) of 70-80 amino acids in the N-terminal region.[96] Each BIR domain coordinates a zinc ion by a histidine and three cystidine residues and resembles a classic zinc finger structure.[97,98] BIR-domains can be further subdivided into type-I BIR domains that lack a peptide binding groove and type-II BIR domains that carry a distinctive hydrophobic cleft.[99] With this cleft, IAPs bind to IAP-binding motifs (IBMs) occurring in caspases and several inhibitory proteins including Smac.[100] The BIR domain is critical for the direct inhibition of caspases since a minimum of one BIR domain is necessary for protein-protein interaction and suppression of Fas-induced apoptosis by XIAP.[101–104] Early biochemical studies revealed that BIR3 is a specific inhibitor for caspase-9 whereas the linker domain between BIR1 and BIR2 inhibits caspase-3 and -7.[105] The human IAP family includes:[106]

- Neuronal apoptosis inhibiting protein (NAIP, BIRC1)
- Cellular IAP1 (cIAP1, BIRC2)
- Cellular IAP2 (cIAP2, BIRC3)
- X-linked IAP (XIAP, BIRC4)
- Survivin (BIRC5)
- Ubiquitin-conjugating BIR domain enzyme (Apollon, BIRC6)
- Melanoma IAP (ML-IAP, BIRC7)
- IAP-linked protein 2 (ILP2, BIRC8)

1.3.2 Molecular structure of IAPs

The most thoroughly characterized mammalian IAPs XIAP, cIAP1 and cIAP2 each contain three BIR domains. In addition, all IAPs, excluding survivin and BIRC6, carry a really interesting new gene (RING)-type ubiquitin protein ligase (E3) at the carboxy-terminus.[106] RING-type E3s recruit different ubiquitin-conjugating enzymes (E2s) and mediate protein ubiquitination.[107–109] In principle, ubiquitination is a regulatory post-translational modification and specifies the fate and function of the modified protein.[108,110] Interaction of the RING domain with E2s requires activation and dimerization of RING prior to protein modification.[111–113] The RING dimerization is a critical activation step for IAPs and exerts a regulatory role for the E3 ligase in order to avoid random substrate ubiquitination. In the absence of a substrate, intramolecular interaction between cIAP1's RING and the BIR3 domain prevents RING exposure and restrains dimerization.[114] Unlike cIAP1 and cIAP2, the RING domain of XIAP lacks BIR-autoinhibition and directly dimerizes and engages E2s without prior activation.[115] A third domain, called caspase recruitment domain (CARD), is found in cIAP1 and cIAP2. Recent research revealed the structure and autoinhibitory function of this domain as a suppressor of the RING-domain's E3 ligase activity.[116]

Several studies support the assertion that, despite the structural homology, XIAP, but not cIAP1 and cIAP2, is the only real endogenous caspase inhibitor.[117,118] XIAP binds caspase-3 and caspase-7 via its BIR2 motif and sterically occlude the active side. Likewise, BIR3-caspase-9 interaction forces caspase-9 monomerization which collapses the caspase's binding pocket and restrains execution of mitochondrial apoptosis.[117] Given that only XIAP directly inhibits caspases, IAPs may be involved in additional cell death-related signaling pathways. This assumption is supported by the fact that gene-expression analysis delineated cIAP1 as a promoter of tumorigenesis, facilitating cancer cell survival.[119] Consistently, emerging evidence indicates that cIAP1's and cIAP2's anti-apoptotic role is based on regulating death receptor-related response following TNF signaling.[120] As outlined above, cIAP1 and cIAP2 are both recruited to complex I after ligation of TNFα to TNFR1 and act together to ubiquitinate RIPK1 via their RING-

domain.[50] This promotes antiapoptotic signaling pathways, including NFκB and MAPK/JNK which stimulate genes involved in proliferation, differentiation and inflammation and restrains proapoptotic signals such as complex IIA-dependent activation of caspase-8.[120] Similarly, IAPs are involved in other signaling networks including inflammation and immunity acting as integrators and molecular switches.[121]

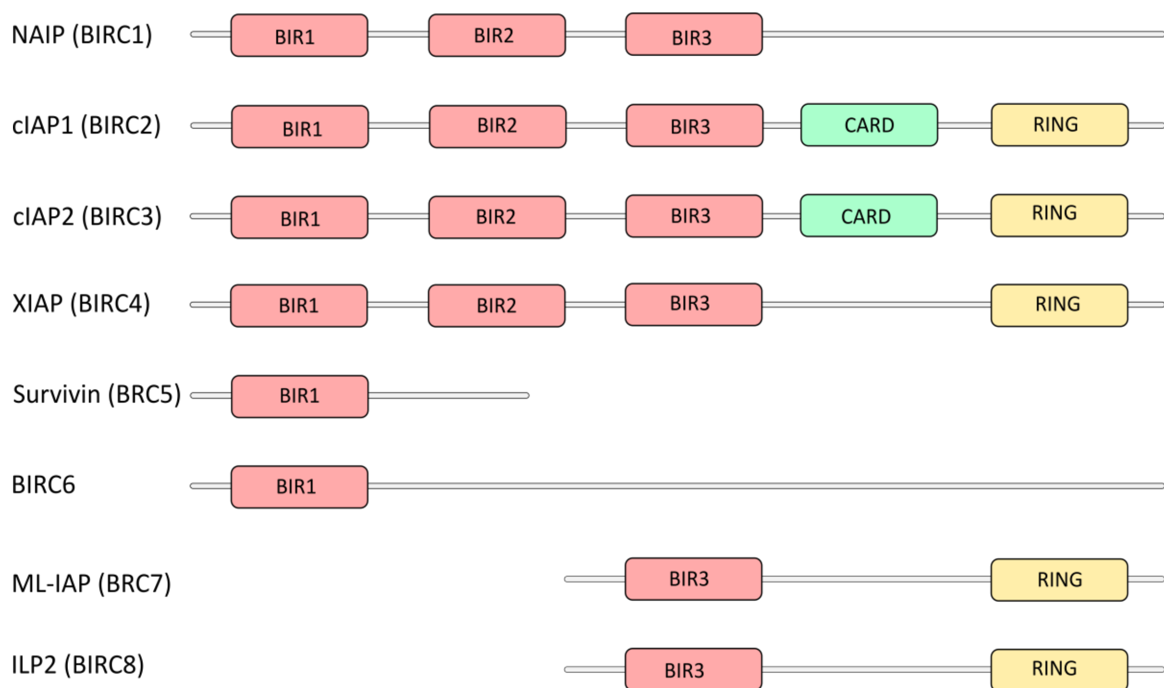

Figure 2 Schematic representation of the seven human IAP family members

Baculoviral repeats (BIR1-3) enable interactions with proteins including caspases and are critical for endogenous inhibition of apoptosis. The RING domain facilitates ubiquitin E3-ligase function and enables IAPs to target proteins for ubiquitin-dependent posttranslational modification. The CARD domain is found in cIAP1 and cIAP2 exclusively and appears to restrain RING-domain activation in an autoregulative manner. This scheme includes the relevant functional domains, any additional structures are left out (adapted and modified from [122])

1.4 Smac mimetics

1.4.1 Structure of Smac mimetics

IAPs, as outlined above, comprise a family of anti-apoptotic proteins that are frequently overexpressed in various human cancers promoting evasion of cell death and cancer cell survival. From the seven human IAPs, only XIAP directly inhibits endogenous caspases and is hence an attractive target for molecular cancer therapies. However, amplification of chromosome 11q22, which harbors both *cIAP1* and *cIAP2* is frequently found in several human malignancies and correlates with chemoresistance and poor outcome.[94,123–128] In 2000, Smac, a small molecule released from the mitochondrial inter membrane space was found to effectively bind and antagonize several IAPs thus preventing caspase inhibition.[129,130] Moreover, several studies have shown that overexpression of Smac sensitizes cells for apoptotic stimuli and low-level expression of Smac correlates with advanced tumor stage and poor prognosis.[131–134] Wild-type Smac is synthesized as a precursor molecule of 239 amino acids and carries a N-terminal 55-residues mitochondrial-targeting sequence (MTS), which is proteolytically removed upon mitochondrial release to expose a N-terminal tetrapeptide (Ala-Val-Pro-Ile/AVPI)-IAP binding side (IBM).[135,136] Mature Smac can homodimerize and bind BIR2 and BIR3 motifs of XIAP via its IBM stretch to release caspase-3, -7 and -8 from its inhibition thus promoting caspase activation and apoptosis.[103,137] Furthermore, topological data suggest that the four-residue peptide AVPI derived from Smac-IBM can bind IAPs with the same affinity as mature Smac and effectively antagonize caspase inhibition.[136] This revelation spurred the development of small molecular IAP-inhibitors, termed Smac mimetics that structurally derive from the N-terminal IBM-motif of mature Smac.[131] In the last decade, several potent and cell-permeable small-molecule Smac mimetics have been designed through extensive chemical modifications of each of the four AVPI residues.[138,139] In addition to monovalent Smac mimetics, bivalent Smac mimetics, composed of two monomeric units connected through a chemical linker, were developed to reproduce the homodimeric character of mature Smac and the concurrent

binding of both BIR2 and BIR3, considerably expanding the efficiency of IAP-targeting agents.[131,140,141] Data yielded for bivalent Smac mimetics in cell death and tumor growth inhibition assays displayed a marked increase in potency compared to monovalent agents, which might be due to the enhanced cIAP dimerization and simultaneous XIAP BIR2- and BIR3-engagement resulting in caspase-3 and -7 activation.[142–145]

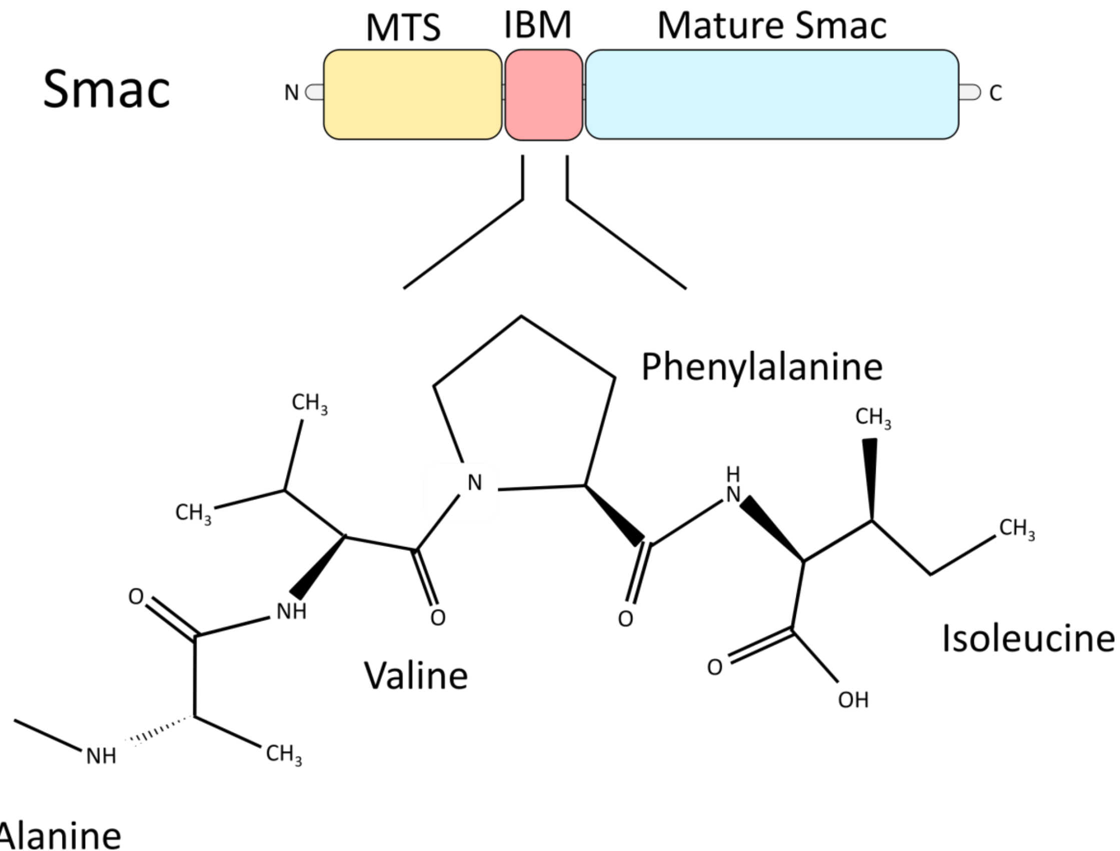

Figure 3 Structure of Smac and N-terminal AVPI-motif.

Smac consists of three functional domains. The N-terminal MTS is proteolytically removed upon release from the mitochondria to expose the Ala-Val-Phe-Ile (AVPI) encompassing IBM which is critical for the binding of Smac to BIR2 and BIR3. The AVPI peptide provides the structural basis for the Smac-derived small molecule IAP inhibitors, termed Smac mimetics, which mimic the binding of Smac to XIAP, cIAP1 and cIAP2 (adapted and modified from [146]).

1.4.2 Inhibition of IAPs

Wild-type Smac and synthetic small molecule IAP inhibitors bind BIR2 motifs of cIAP1 and cIAP2 via the AVPI binding motif. This enhances the IAP-RING domain's E3 ligase-activity to facilitate autoubiquitination and proteasomal degradation of cIAP1 and cIAP2.[147,148] However, this mechanism has been exclusively described for cIAP1 and cIAP2 but not for XIAP.[147] cIAP1 and cIAP2 cooperate in the non-canonical NFκB pathway

to promote ubiquitin-dependent proteasomal degradation of NIK. Thus Smac-imposed degradation of cIAP1 and cIAP2 causes accumulation of NIK and subsequent activation of non-canonical NFκB signaling.[149,150] Moreover, several studies revealed additional activation of canonical NFκB signaling as indicated by IκBα degradation and p100 processing.[150,151] Because binding of RIPK1 to TNFR1 is known to induce canonical NFκB signaling through its ability to recruit the IKKβ containing complex, it is argued that degradation of cIAP1 induces canonical NFκB signaling via the increased recruitment of RIPK1 to TNFR1.[151,152] However, the mechanisms for the activation of the canonical NFκB pathway are not fully elucidated.

It is generally agreed that Smac- and Smac mimetic-induced degradation of cIAP1 and cIAP2 prior to NIK accumulation and activation of RelB:p50 drives TNFα production and sensitizes cells to TNFα-induced apoptosis, despite NFκBs role in cell death evasion.[150,151] This autocrine/paracrine production of TNFα has been shown to facilitate caspase-8 activation in a RIPK1-dependent manner. In the absence of small molecular IAP inhibitors, RIPK1 can be polyubiquitinated by cIAP1 and cIAP2 thereby serving as the key scaffold molecule for the assembly of pro-survival complex I.[50,51] In cIAP-depleted cells, deubiquitinated RIPK1 is released from complex I and transits into a cell death-inducing complex capable of recruiting FADD and procaspase-8.[53,153] This RIPK1:FADD:procaspase-8 complex has the ability to activate caspase-8 in a TNFα-dependent manner thus initiating apoptosis.[154] Current *in vitro* research on glioma cells supports the critical role for the RIPK1:FADD:caspase-8 complex and NFκB activation for Smac mimetic-induced apoptosis. Genetic inhibition of NFκB through dominant negative IκBα-superrepressor (SR) cells significantly decreases Smac mimetic-induced radiosensitization.[155] Furthermore, knock down of RIPK1 by small interfering RNA (siRNA) notably reduces Smac mimetic-induced caspase-8 activation and apoptosis.[156] A recent study by our group showed that Smac mimetics stimulate NFκB-dependent upregulation of death receptor DR5 prior to the formation of the cytosolic RIPK1:FADD:caspase-8 complex to induce apoptosis independent of the soluble form of the receptor ligand TRAIL.[157] It is noteworthy that the finding of the study suggests an additional, TNF-independent mechanism for Smac mimetic-induced apoptosis.

1.4.3 Smac mimetic-based combination protocols

In line with the notion that Smac mimetics can sensitize cells to apoptotic stimuli, they were extensively studied in combination protocols together with cytotoxic agents including standard of care chemotherapeutics, death receptor ligands and cytokines. In fact, Smac mimatics promote TNFα-dependent cell death but insufficient levels of TNFα hamper efficient induction of apoptosis. Furthermore, cancer cells treated with Smac mimatics can harness molecular feedback loops in order to increase cIAP2 expression which compensate for the low levels of cIAP1.[158] Several combination studies provide evidence that the combination of Smac mimetics and cytotoxic agents can be used to exacerbate caspase activation and cancer cell death. The data suggests that this effect is mediated through multifaceted mechanisms involving enhanced paracrine TNFα-loops and NFκB activation.[159,160]

Combining Smac mimetics with cytotoxic agents is hence a conceivable approach to identify promising therapy regimens. Based on this approach, small molecular IAP inhibitors have been proven to sensitize a broad spectrum of human cancers, including pancreatic, lung, colon, breast and skin cancer as well as acute leukemia towards chemotherapeutics such as doxorubicin, cisplatin, 5-fluoruracil and vinorelbine.[131,160–164] In addition to chemotherapeutics, small molecular IAP antagonists have been extensively studied in combination with death receptor ligands, including TRAIL and TNFα, epidermal growth factor receptor antibodies such as trastuzumab and small molecular kinase inhibitors such as lapatinib or gefitinib resulting in an augmentation of the antitumor effects of these agents.[131,165–170]

1.4.4 Smac mimetics in clinical trials

Currently, several Smac mimetic-based combination protocols are under evaluation in phase I studies to document the clinical applicability, safety and tolerability as

therapeutics in patients with advanced solid tumors and hematological malignancies.[171,172] The initial clinical data provide evidence that small molecular IAP antagonists are well tolerated and have notable on-target antitumor activity as single agents and in combination therapies.[136] Further research is inevitable to gain broader insight into the pharmacokinetics and pharmacodynamics of Smac mimetics and to extend the spectrum of biomarkers suitable for predicting the clinical response of patients with Smac mimetic-based therapies.[173]

In conclusion, the inhibition of IAPs by small molecular Smac-derived agents, termed Smac mimetics, is a promising approach to target elevated levels of IAP and enhance cancer cell susceptibility towards apoptotic stimuli.

1 Introduction

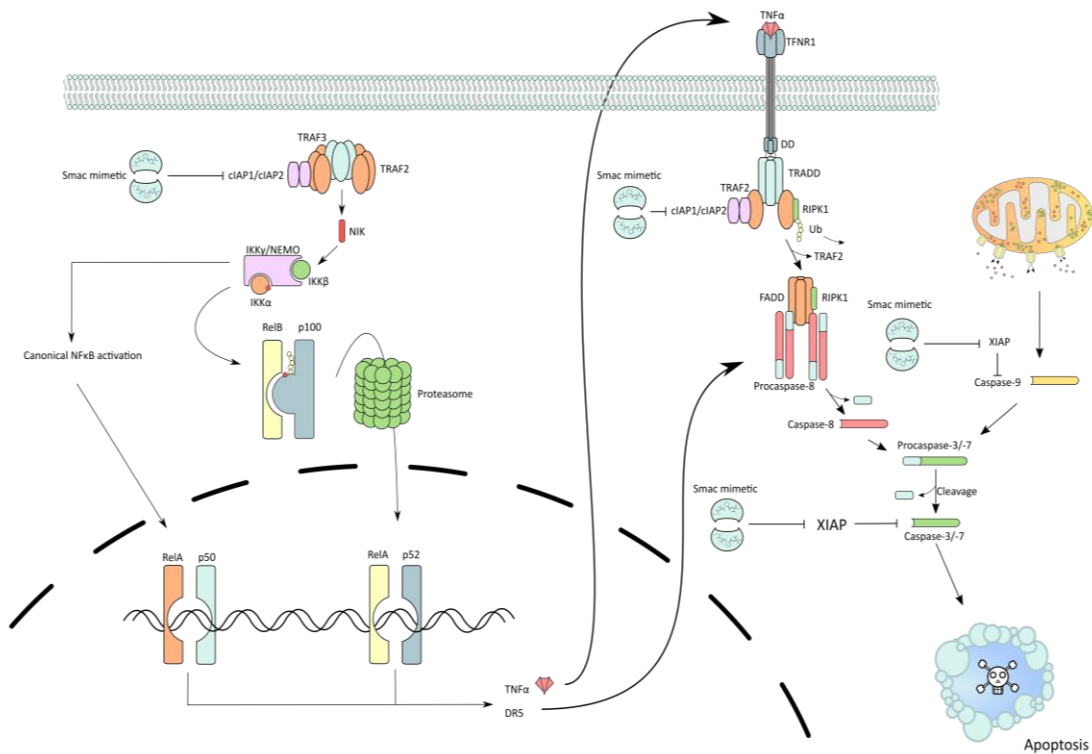

Figure 4 Overview of the cellular effects of Smac mimetics.

In unstimulated cells, high levels of cIAP1 and cIAP2 restrain NIK accumulation through constitutive ubiquitination-dependent proteasomal degradation. Smac mimetics promote NFκB activation by depleting endogenous cIAP1 and cIAP2 thus facilitating NIK accumulation and proteasomal p100 processing. Activated NFκB transcription factors enhance expression of proapoptotic genes including TNFα and DR5. Upon inhibition of cIAP1/2-dependent RIPK1 ubiquitination, TNFα can induce formation of a proapoptotic complex composed of RIPK1, FADD and procaspase-8 in a paracrine/autocrine manner. The complex eventually cleaves procaspase-8 into active caspase-8 inducing apoptosis. Furthermore, Smac mimetics can bind XIAP and inhibit interaction with caspase-3, -7 and -9 further enhancing the ability to induce TNFα-dependent apoptosis.

1.5 NFκB

1.5.1 The NFκB family

The nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB)-family is a group of five structurally related and highly conserved signal-responsive transcription factors (TFs): Rel (c-Rel), RelA (p65), RelB, NFκB1 (p50 and its precursor p105) and NFκB2 (p52 and its precursor p100).[174,175] These TFs can be further subdivided based on their cellular synthesis and mode of activation. Members of the Rel subfamily, including Rel, RelA and RelB are synthesized in their mature form and can bind DNA as dimers via their rel homology domain (RHD) to regulate gene transcription.[176,177] The second group, termed the NFκB subfamily, is synthesized as large precursors (p100 and p105) with an N-terminal RHD and several C-terminal inhibitory ankyrin repeats that prevent the unprocessed precursor from dimerization and subsequent DNA binding.[174,177,178] Upon activation, the precursors are polyubiquitinated and posttranslationally cleaved into their mature form.[176] The proteolytic processing separates the N-terminal RHD from the ankyrin repeats and produces the active p50- and p52-DNA-binding subunits.[179] Homo- and heterodimers of NFκB family members rapidly bind to cognate κB-motifs on the DNA and modulate a plethora of cellular functions including cell survival and proliferation, differentiation, apoptosis and innate and adaptive immunity. Many of those pathways are involved in oncogenesis and a large body of evidence suggests NFκB activation as fundamental for the development of several malignancies.[180,181]

1.5.2 The canonical NFκB pathway

In principle, activation of NFκB family members is controlled by two distinct signaling pathways, called the canonical and the non-canonical pathway.[182] Activation of the pathways results either in the release of NFκB from its inhibitor (IκBα, canonical) or the proteolytical processing of the premature p100 to p52 (non-canonical). Activation of the

canonical as well as the non-canonical pathway follows various extracellular stimuli. The best understood mechanisms of canonical activation include binding of liposaccharides (LPS) to Toll-like receptors (TLRs), mainly TLR4, binding of cytokine Interleukin-1 to its receptor IL-1R and the ligation of soluble TNFα to TNFR1.[46,183,184] Like TNFRs, active TLRs and IL-1R can form intracellular domains (TLR and IL-1R homology domain, TIR) and recruit adapter proteins to yield molecular signaling platforms.[185,186] Activation of NFκB by members of the TNFR-family requires recruitment of TRADD, TRAF2 and/or 5 and RIPK1 to TNFR1's DD forming complex I (as addressed earlier in chapter 2: Signaling pathways of apoptosis).[187,188] RIPK1, along with TRAF, is an essential scaffold protein for the complex and is required for the activation of downstream signaling proteins.[49,189] Complex I serves as a docking side for IκBα kinase β (IKKβ), the main convergence point for NFκB-activating signal transduction pathways. This brings kinase and substrate into close proximity and facilitates IKKβ phosphorylation.[183,190,191] IKKβ is part of a large, 700-900 kDa oligomeric protein complex consisting of IKKα, IKKβ and NFκB essential modulator (NEMO).[183,192,193] IKKα and IKKβ share a similar structure and each contain a N-terminal kinase domain required for phosphorylation of the NFκB inhibitor IκBα. In contrast, NEMO has no catalytic activity and acts as a regulatory subunit for the complex.[193,194] TNF-induced activation of IKKβ results in the rapid phosphorylation and proteasomal degradation of IκBα within a couple of minutes.[195] To bind κB motifs and regulate transcriptional programs, NFκB TFs form dimers via their RHD. In a quiescent cell, the canonical heterodimer RelA:p50 is bound to the IκBα.[196] The IκB-family consists of nine members each containing at least six ankyrin repeats to block NFκB TFs DNA binding side and control cytosolic localization of the inactive complex.[197–199] Phosphorylation of IκBα at a conserved serine residue results in polyubiquitination via an ubiquitin E3-ligase complex followed by rapid proteasomal degradation.[200] This shifts the balance between cytosolic and nuclear localization of RelA:p50 dimers favoring the latter and allows sustained binding to κB sites on NFκB-responsive promotors.[182]

1.5.3 The non-canonical NFκB pathway

The alternative, non-canonical NFκB pathway primarily targets the RelB:p52 complex. RelB lacks classical IκB-dependent regulation but is sequestered in the cytosol through dimerization with the precursor protein p100. In order to fully function as a TF, the proteasome processes the separation of p100 C-terminal IκB-like ankyrin-repeats from the p52 subunit containing the DNA binding RHD.[201,202] The non-canonical pathway is activated by a small number of extracellular receptors. Similar to the canonical pathway, the best studied receptors are members of the TNF-receptor family, including lymphotoxin β receptor (LTβR), B cell activating factor-receptor (BAFF-R) and receptor activator of NFκB (RANK).[203–205] Activation of either of this receptors converges at the level of NFκB inducing kinase (NIK). NIK is a MAP-3 kinase-related kinase and considered as the key switch in non-canonical NFκB signaling.[206,207] Upon activation, NIK and IKKα cooperate to induce site-specific phosphorylation and subsequent E3 ligase-dependent ubiquitination of p100.[208–210] The ubiquitinated p100 is recognized by the proteasome which removes the inhibitory C-terminus releasing mature p52.[211] Although the exact mechanisms by which receptor activation results in NIK accumulation and p100 processing remains incompletely understood, emerging evidence suggests the requirement for cIAP1/2-dependent degradation of TRAF-family proteins during the induction of non-canonical NFκB signaling. In resting cells, NIK is constantly expressed and rapidly degraded via proteasomes keeping NIK activity on a low basal level.[212,213] Members of the TRAF-family, particularly TRAF2 and TRAF3, act as adapters and recruit cIAP1, cIAP2 and NIK into a stable complex. This complex acts as a cIAP-mediated ubiquitin E3-ligase that polyubiquitinates NIK and induces its rapid turnover via proteasomal degradation.[214,215] Receptor stimulation induces polyubiquitination of cIAP1/2 and redirects the E3-ligase activity towards the TRAF-adapter proteins triggering their degradation.[214,216] This disrupts the complex and prevents NIK from cIAP1/2-dependent degradation allowing NIK accumulation and p100 processing.[213,217,218] Genetic evidence suggests that the non-canonical pathway is far more selective compared to the canonical NFκB pathway.[219] It is well established that non-canonical

NFκB signaling plays a pivotal role in the organogenesis of secondary lymphoid organs, regulation of T-cell differentiation and osteogenesis.[220,221]

1.5.4 NFκB in tumorigenesis

Since the initial discovery of the oncogenic RelA homologue v-Rel, an oncogenic potential of the NFκB pathway was taken into consideration.[222,223] So far, direct mutations of NFκB have been detected primarily in lymphoid malignancies (e.g. Hodgkin's and non-Hodgkin's lymphoma, lymphatic leukemia) but rarely in solid tumors.[224–227] Furthermore, constitutively elevated NFκB levels are a common feature found in a broad variety of human malignancies. They seem to act as important co-factors enabling the cells to withstand apoptosis (e.g. via enhanced expression of IAPs and Bcl-2 proteins) and outperform the host's immune system which contributes to tumor progression.[228–230] However, there is recent evidence that the canonical and non-canonical pathway can, under certain circumstances, mediate pro-apoptotic and growth-inhibitory effects. NFκB-mediated susceptibility to apoptosis can be induced by transcription of tumor suppressor p53-encoding genes and subsequent increased levels of p53 and p53 target genes.[231,232]

In summary, the mammalian NFκB TF-family consists of five proteins that can bind to κB DNA motifs as homo- and heterodimers and regulate transcriptional programs involved in a large variety of cellular functions such as proliferation, apoptosis, inflammation and immunity. Aberrant regulation of NFκB pathways is found in the majority of malignancies and sustains cell survival, cancer immune escape and tumor progression. However, current research revealed novel convergence between NFκB signaling and p53-dependent growth-inhibitory pathways and NFκB-dependent susceptibility towards apoptosis. Nonetheless, further research is necessary to elucidate the underlying mechanisms as well as the physiological and pathological implications of these new findings.

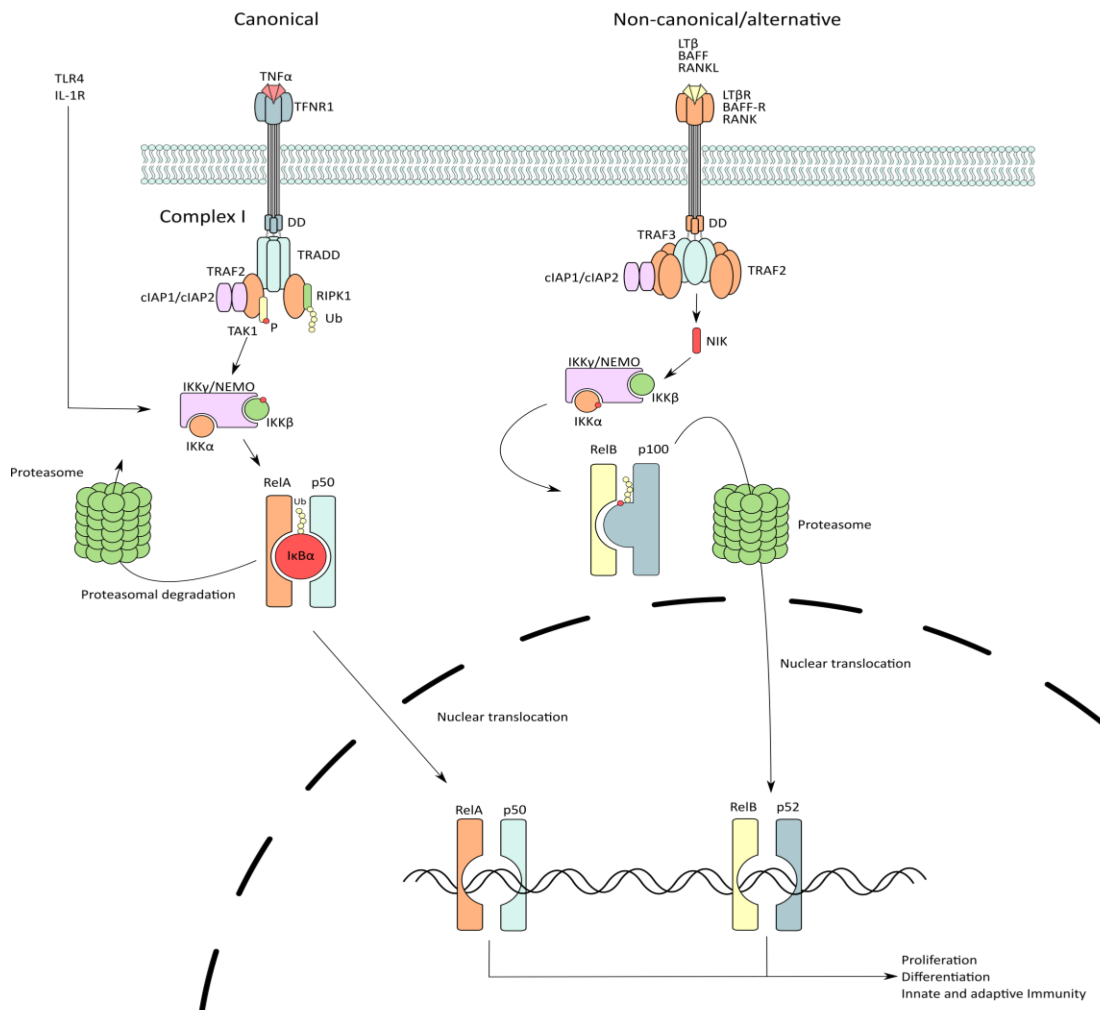

Figure 5 Overview of the NFκB signaling pathway.

Activation of NFκB family members is controlled by two distinct signaling pathways, termed the canonical and the non-canonical pathway. TNFα-dependent formation of complex I facilitates along with several other human cytokine receptors, phosphorylation and subsequent activation of the IKKβ-containing oligomeric kinase complex. Activation of IKKβ results in the rapid proteasomal degradation of IκBα and concomitant liberation of RelA:p50 DNA-binding side sustaining nuclear translocation and regulation of transcriptional programs. The non-canonical NFκB pathway is activated by a wide variety of extracellular receptors including LTβR, BAFF-R and RANK. Activation of either receptors converges at the level of NIK activation which induces ubiquitin-dependent proteasomal processing of p100 into mature p52. The novel

1 Introduction

RelB:p52 complex translocates into the nucleus and regulate target gene transcription involved in proliferation, differentiation and innate and adaptive immunity.

1.6 Interferon α

1.6.1 Taxonomy and biological properties of Interferons

Interferons (IFNs) represent a diverse group of thoroughly characterized human cytokines with antiviral properties originally discovered over six decades ago.[233] Since then, extensive studies granted comprehensive insight into the structure, function and signaling pathways and revealed a pivotal role of IFNs in innate and adaptive immunity, tumor surveillance, cell differentiation and apoptosis.[234,235] Interferons are grouped into three types (type I-III) based on gene location, biochemical properties and receptor specificity.[236,237] Type I IFNs consist of five classes found in humans: IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω. Type II consists of IFN-γ only while type III IFNs, also designated as IFN-like cytokines, consist of four additional members (interleukin-29/IFNλ1, interleukin-28A/IFNλ2, interleukin-28B/IFNλ3 and IFNλ4).[238–241] Type I IFNs display a high level of structural redundancy and engage the same cognate cell-surface receptor for signal transduction, known as the type I/α IFN-receptor (IFNAR).[237] IFNAR consists of two distinct subunits, called IFNAR1 and IFNAR2, each interacting with a member of the Janus activated kinase (JAK)-family.[242–247] The intracellular domain of IFNAR1 is constitutively associated with tyrosine kinase 2 (TYK2) whereas IFNAR2 is associated with JAK1.[239,246,248] Binding of the monomeric IFNα to the receptor results in rapid autophosphorylation and concomitant activation of TYK2 and JAK1.[246] Active TYK2/JAK1 facilitates reciprocal phosphorylation of tyrosine residues on the cytoplasmic tail of the receptor.[249,250] This receptor-phosphorylation creates a docking side for members of the signal transducer and activator of transcription (STAT)-family, namely STAT1 and STAT2 that are involved in the regulation of many aspects of cellular growth, survival and differentiation.[251,252] The STAT-family of TFs comprises seven mammalian members (STAT 1-4, 5A, 5B, 6) that reside in the cytoplasm in absence of receptor stimulation.[253] In response to receptor ligation, STATs are recruited to the cytoplasmic docking side through specific binding between the STAT's Src homology 2 (SH2)-domain and the receptor's phosphotyrosine residues.[254] Ligation of type I and type II IFNs facilitates

recruitment and JAK-dependent C-terminal phosphorylation of STAT1 and STAT2.[255] Prior to nuclear translocation, phosphorylated STAT1:STAT2 recruit interferon regulatory factor 9 (IRF9) assembling a tertiary complex termed interferon-stimulated gene factor 3 (ISGF3).[246,256] The association with IRF9 via its C-terminal IRF-associated domain (IAD) is a crucial step in the activation of ISGF3 and results in the recognition of a distinct DNA response element called ISRE (interferon-stimulated response element) which resides in the promotor of certain IFN-stimulated genes (ISGs). Members of the IRF-family are important secondary transcription mediators of endogenous IFN-induced signaling pathways.[257] All nine human IRF members (IRF1-9) are modular proteins and share a well conserved N-terminal homology which contains the DNA binding domain (DBD) of about 120 amino acids and facilitates recognition of specific repetitive DNA-sequences.[258,259] There is evidence that each member of the IRF-family exerts distinct roles in biological processes such as hematopoiesis, cell growth regulation, host defense and even metabolism.[257,260] Subsequent studies linked aberrant IRF signaling to numerous diseases including autoimmune disorders, allergies and cancer.[257,261–264] Aside from host immunity, IRFs, primarily IRF1, have a critical function in the regulation and prevention of oncogenesis as tumor suppressors.[265] Studies performed on IRF-deficient cells showed insufficient genotoxic-induced cell-cycle arrest, impaired DNA repair mechanisms and resistance to DNA damage-induced apoptosis.[263,266] Upon DNA damage, IRF1 cooperates with another pivotal tumor suppressor p53 to transcriptional induce the gene encoding p21$^{WARF1/CIP1}$. Moreover, TNF-α and IFN-γ-induced IRF1-activation can promote apoptosis in several cell lines (e.g. T-lymphocytes, breast cancer cells) and transcriptionally induce genes encoding caspase-1, caspase-7, caspase-8, FasL and TRAIL thus sensitizing cells to apoptotic stimuli and counteracting anti-apoptotic pathways including NFκB.[267–272]

To sum up, IFNs represent a diverse group of human cytokines with essential roles in adaptive and innate immunity, cell differentiation and apoptosis. Members of the IRF family are IFN-induced TFs and have distinct roles in biological processes. IRF1, the firstly discovered IRF, acts as a tumor suppressor and can transcriptionally induce genes encoding CDK-inhibitors (p21$^{WARF1/CIP1}$), caspases (e.g. caspase-8), and anti-proliferative

signaling proteins (e.g. TRAIL and FasL). Moreover, aberrant IFN signaling and IRF deficiency is linked to numerous human diseases including autoimmune disorders, allergies and cancer.

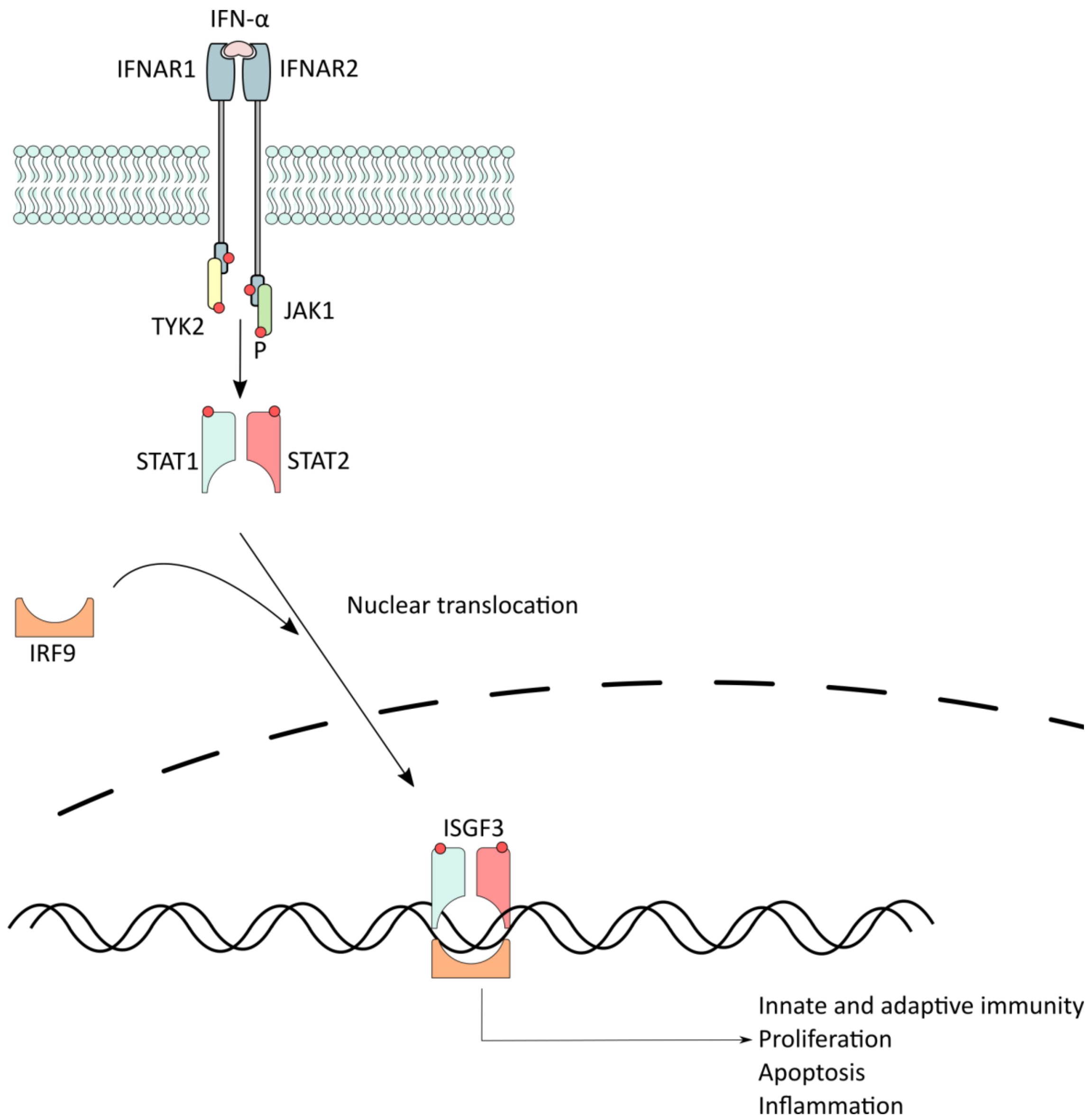

Figure 6 Overview of the IFNα signaling pathway

Binding of type I interferons, IFN-α for instance, to the cell-surface receptor IFNAR1/IFNAR2 promotes dimerization and phosphorylation-dependent activation of JAK-STAT signaling. Activated STAT1:STAT2 recruit IRF9 into a complex termed ISGF3 and translocate into the nucleus prior to binding and regulation of IFN-responsive nuclear target patterns. IFN signaling exerts distinct roles in biological processes including hematopoiesis, cell growth regulation and host defense.

1.7 Aims of the study

The primary aim of this study is

1. To investigate and delineate the antitumoral activity of the small molecular Smac mimetic BV6 in combination with IFNα in glioblastoma multiforme cell lines.

To achieve this objective, we are first going to explore the antitumoral activity of BV6 and IFNα as single and combination treatment in several GBM cell lines. To evaluate the biological activity of the agents and identify suitable concentrations, we will use apoptosis-specific cell death assays, including assessment of DNA fragmentation and loss of cell viability.

Secondary aims of the study are

2. To investigate the molecular mechanism underlying the BV6/IFNα treatment paying particular attention to the role of canonical and non-canonical NFκB signaling as well as death inducing cytokines, including TNFα and TRAIL.
3. To investigate the contribution of NFκB to the antitumoral activity using western blotting to assess activation of NFκB key molecules such as NIK, IκBα and p100
4. To explore the relevance of NFκB signaling for BV6/IFNα-induced cell death by constitutive inhibition of the canonical pathway
5. To analyze the impact of pharmacological TNFα and TRAIL inhibition on the BV6/IFNα cotreatment

The results of this study will have important implications for the development of Smac mimetic-based treatment strategies for GBM.

1 Introduction

2 MATERIAL AND METHODS

2.1 Material

2.1.1 Cell lines

All cell lines used in this research project derived from established human glioblastoma multiforme cell lines

A172	ATCC
U87MG	ATCC
T98G	ATCC
U118	ATCC
LN229	ATCC
A172 IκBα EV/SR	generated and kindly provided by I. Eckhardt
A172 reporter cells	generated and kindly provided by I. Eckhardt

For the generation of A172 and U87MG IκBα EV/SR cells, pCFG5-IEGZ control vector and pCFG5-IEGZ IκBα-S(32, 36)A were used (kindly provided by B. Baumann). For the generation of A172 NFκB reporter cells, the pTRH1 NF-κB-EGFP vector was used (kindly provided by J. Silke).

2.1.2 Cell culture and cultivation

Dulbecco's modified eagle medium (DMEM)	Life Technologies, Inc., Eggenstein, Germany
Trypsin-EDTA, 0.05%	Life Technologies, Inc., Eggenstein, Germany
Fetal calf serum (FCS)	Biochrom, Berlin, Germany
Sodium Pyruvate (1mM)	Invitrogen, Karlsruhe, Germany
Penicilline/Streptomycin	Invitrogen, Karlsruhe, Germany

2.1.3 Cell death induction and inhibition

Smac mimetic BV6	Genentech (San Francisco, USA)
Interferon-α 2a human	Sigma-Aldrich (St. Louis, USA)
Enbrel® (Etanercept)	Pfizer (New York, USA)
TRAIL-blocking antibody 2E5	Enzo Life Sciences (Lörrach, Germany)
zVAD.fmk	Bachem (Bubendorf, Switzerland)

2.1.4 General chemicals

Bromophenol blue	Carl Roth (Karlsruhe, Germany)
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich (St. Louis, USA)
Disodium phosphate (NA_2HPO_4)	Carl Roth (Karlsruhe, Germany)
Ethanol	Carl Roth (Karlsruhe, Germany)
Glycerol	Carl Roth (Karlsruhe, Germany)

Glycin	Carl Roth (Karlsruhe, Germany)
Isopropanol	Sigma-Aldrich (St. Louis, USA)
Potassium dihydrogen phosphate (KH_2PO_4)	Carl Roth (Karlsruhe, Germany)
Methanol	Carl Roth (Karlsruhe, Germany)
Sodium chloride (NaCl)	Carl Roth (Karlsruhe, Germany)
Propidium iodide	Sigma-Aldrich (St. Louis, USA)
Tris	Carl Roth (Karlsruhe, Germany)
Trypan blue	Invitrogen (Carlsbad, USA)
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Sigma-Aldrich (St. Louis, USA)

2.1.5 Electrophoresis and Western Blotting

Ammonium persulfate 10% (APS)	Carl Roth (Karlsruhe, Germany)
Bovine serum albumin (BSA)	Carl Roth (Karlsruhe, Germany)
Dithiothreitol (DTT)	Calbiochem MerckMillipore (Darmstadt, Germany)
Filter paper	Carl Roth (Karlsruhe,Germany)
Nitrocellulose membrane Hybond ECL 0.45µm	Amersham Bioscience (Amersham, UK)
Hyperfilm ECL	Amersham Bioscience (Amersham, UK)
Milk powder	Carl Roth (Karlsruhe, Germany)
PageRuler plus prestained protein ladder	Fermentas (Burlington, USA)
Pierce® ECL Western Blot detection reagent	Thermo Fisher Scientific (Waltham, USA)

Protease inhibitor cocktail	Carl Roth (Karlsruhe, Germany)
Roenteroll HC x-ray developer	TETENAL (Somerville, UK)
Rotiphorese® gel 30	Carl Roth (Karlsruhe, Germany)
Sodium dodecyl sulfate (SDS)	Carl Roth (Karlsruhe, Germany)
X-ray developer solution	TETENAL (Somerville, UK)
Superfix MRP x-ray fixer solution	TETENAL (Somerville, UK)
Tetramethylethylenediamine (TEMED)	Carl Roth (Karlsruhe, Germany)
Triton X-100	Carl Roth (Karlsruhe, Germany)
Tween® 20	Carl Roth (Karlsruhe, Germany)

2.1.6 Buffers and solutions

12% separation gel for the SDS-polyacrylamide gel electrophoresis	0.1% ammonium persulfate, 0.01% TEMED, 42% acrylamide/ bisacrylamide, 0.1% SDS, 25% Tris (1.5 M; pH 8.8), 33% aqua dest.
5% collection gel for the SDS-polyacrylamide gel electrophoresis	0.1% ammonium persulfate, 0.01% TEMED, 17% acrylamide/ bisacrylamide, 0.1% SDS, 12.5% Tris (1.5 M; pH 6.8), 68% aqua dest.
Blocking buffer	500 ml PBST, 25 g fat free dry milk powder
Blotting buffer	48 mM Tris base, 39 mM glycin, 0.1% SDS, 20% methanol
BSA sodium azide	2% BSA, 0.02% sodium azide, 500 ml PBST

BSA standard serial dilution	Stock solution 2 mg/ml BSA, solubilized in H_2O in concentration of 0.025; 0.125; 0.25; 0.5; 0.75; 1; 1.5; 2 mg/ml
DPBS (1x)	PAA Laboratories (Egelsbach, Austria)
Running buffer for the SDS-polyacrylamide gel electrophoresis	125 mM Tris base, 1.25 M SDS 10%, 10% glycin, 1 l H_2O
Lysis buffer	30 mM Tris-HCL (pH 7.5), 150 mM NaCl, 1% Triton-X 100, 10% glycerol, 200 ml H_2O, protease inhibitor cocktail, 2 mM DTT, 200 µM PMSF, 1 mM Na_3VO_4, 10 nM okadaic acid, 1 mM β-glycerol phosphate, 1 ml EDTA, 50 mM sodium fluoride
Nicoletti buffer	50 µg/ml propidium iodide, 0.1% trisodium citrate, 0.4% Triton-X 100
PBS (1x)	140 mM Nacl, 2.7 mM KCl, 3.2 mM KH_2PO_4, 1.5 mM $Na_2HPO_4 \cdot 2 H_2O$, 1 l H_2O
PBST	0.1% Tween® 20 in 1x PBS, dilution 1:1000
SDS loading buffer	TrisBase (1 M; pH 6.8), 5% glycerol, 1% SDS, 2 mM DTT, 0.01 mg/ml bromophenol blue

2.1.7 Antibodies

Primary western blotting antibodies were diluted in 2% BSA/PBS, 0.02% sodium azide and 0.1% Tween®.

Antibody	Species	Solution	Supplier
anti-caspase 3	rabbit	1:1000	Cell Signaling Technology (Danvers, USA)
anti-caspase 8	mouse	1:1000	Enzo life sciences (New York, USA)
anti-caspase 9	rabbit	1:1000	Cell Signaling Technology (Danvers, USA)
anti-cIAP1	goat	1:1000	R&D Systems (Minneapolis, USA)
anti-cIAP2	rat	1:1000	Enzo life sciences (New York, USA)
anti-GAPDH	mouse	1:5000	HyTest (Turku, Finland)
anti-RIPK1	rabbit	1:1000	Santa Cruz biotechnology (Dallas, USA)
anti-RIPK3	rabbit	1:1000	Imgenex (San Diego, USA)
anti-TNFR1	mouse	1:1000	Santa Cruz biotechnology (Dallas, USA
anti-XIAP	mouse	1:1000	BD Biosciences (Franklin Lakes, USA)
anti-β-Actin	mouse	1:10000	Sigma-Aldrich (St. Louis, USA)

Secondary western blotting antibodies were diluted in 5% milk/PBST

Antibody	Solution	Supplier
anti-mouse-IgG, conjugated with horseradish peroxodase (HRP), goat	1:5000	Santa Cruz biotechnology (Dallas, USA)
anti-rabbit-IgG, conj. with HRP, goat	1:5000	Santa Cruz biotechnology (Dallas, USA)
anti-rat-IgG, conj. with HRP, goat	1:5000	Santa Cruz biotechnology (Dallas, USA)
Anti-mouse infrared-green	1:10000	Li-COR Bioscience (Madrid, Spain)
Anti-rabbit infrared-green	1:10000	Li-COR Bioscience (Madrid, Spain)
Anti-goat infrared-green	1:10000	Li-COR Bioscience (Madrid, Spain)

2.1.8 Plastic materials

FACS tubes	BD Biosciences (Franklin Lakes, USA)
Falcon tubes	Greiner Bio-One (Kremsmünster, Austria)
Combitips (0.5 ml; 1 ml; 2.5 ml; 5 ml; 10 ml)	Eppendorf (Hamburg, Germany)
Optical 96 well reaction plate	Applied Biosystems (Foster City, USA)
Pipette tips (10 µl, 200 µl, 1000 µl)	Starlab (Brüssel, Belgium)
Reaction tube (0.5 ml; 1.5 ml)	Starlab (Brüssel, Belgium)
Syringe	B.Braun (Melsungen, Germany)
Sterile pipettes (5 ml, 10 ml, 25 ml)	Greiner Bio-One (Kremsmünster, Austria)

Cell culture flask (25 cm^2, 75 cm^2)	Greiner Bio-One (Kremsmünster, Austria)
Cell culture plate (6, 24, 96-well)	Greiner Bio-One (Kremsmünster, Austria)

2.1.9 Kits

Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific (Waltham, USA)
Venor GeM mykoplasma PCR detection kit	Minerva Biolabs (Berlin, Germany)

2.1.10 Equipment and tools

Autoclave Systec V150	Systec (Wettenberg, Germany)
Balance 440-47N	KERN (Kingston, UK)
Blotting chamber	BioRad (Hercules, USA)
Flow cytometer FACSCanto II operating with FACSDIVA software	BD Biosciences (Franklin Lakes, USA)
Electrophoresis power adapter	BioRad (Hercules, USA)
Heating block thermo mixer comfort	Eppendorf (Hamburg, Germany)
Infinite®M200	TECAN (Männdorf, Switzerland)
Infrared Odyssey® imaging system	Li-COR Bioscience (Madrid, Spain)
Incubator MCO-19AIC	Sanyo (Moriguchi, Japan)
Microscope IX71	Olympus (Tokyo, Japan)
Pipette	Eppendorf (Hamburg, Germany)
Pipet aid Pipetboy® acu	Eppendorf (Hamburg, Germany)

Rolling shaker	Ratek (Boronia, Australia)
Shaker	Caterpillar (Peoria, USA)
Sunrise™ operating with Magellan™ software	TECAN (Männdorf, Switzerland)
Tabletop centrifuge	Carl Roth (Karlsruhe, Germany)
Vortexer	Velp Scientifica (Usmate, Italy)
Laboratory water bath	Medingen (Rochester, USA)
WTW pH meter	Norfab (Trondheim, Norway)
Centrifuge Micro 200R	Hettich (Tuttlingen, Germany)
Centrifuge ROTIXA 50RS	Hettich (Tuttlingen, Germany)

2.2. Cell culture

Splitting, seeding and treatment of the cells was done under sterile conditions in a laminar flow hood. All cell lines were routinely checked for mycoplasma contamination using the Venor GeM mycoplasma PCR detection kit according to the manufacturer's instruction.

2.2.1 Cell cultivation and splitting

Cells were cultured in DMEM medium (Life technologies, Inc., Eggstein, Germany) supplemented with 10% fetal calf serum (FCS) (Biochrom, Berlin, Germany), 1% penicillin/streptomycin (Invitrogen, Karlsruhe, Germany) and 1% sodium pyruvate. The cells were cultivated in 75 cm² cell culture flasks for adherent cells and incubated at 37° C, 5% CO_2 and regulated humidity. The cultivated cells were routinely splitted after 3-4 days and transferred into new cell culture flasks. To this end, cells were rinsed with 2 ml PBS and 2 ml Trypsin were added and evenly distributed. After incubation at 37°C for 5

minutes, 8 ml of media was added and the dilution was transferred into a tube. Split ratio was adapted to cell growth rate, density and future use.

2.2.2 Cell seeding and treatment

For cell harvesting, the old medium was aspirated off and cells were rinsed with 2 ml PBS. The PBS was aspirated off, 2 ml Trypsin was added and evenly distributed with gentle rotation. After incubation at 37° C for 5 minutes, 8 ml of medium was added to the cell culture flask and the dilution was transferred into a tube. A 20 µl sample of the cell suspension was taken and stained with 60 µl trypan blue. Trypan blue can permeate the disintegrated membrane of dead cells while it is excluded from viable cells. The number of viable cells in the suspension was determined by counting the number of unstained cells in the four outer squares of a Neubauer cell counting chamber under the microscope. The concentration of viable cells was calculated by applying the following formula:

$$Concentration\ \left[\frac{cells}{ml}\right] = \frac{number\ of\ cells * dilution\ factor * 10^4}{number\ of\ squares\ counted}$$

The dilution factor is the ratio of trypan blue to cell suspension. Cells were then seeded with a concentration of $3*10^5$ cells/ml. For the MTT cell viability assay, cells were seeded in a 96 well plate with 100 µl cell suspension per well. For the flow cytometry analysis, cells were seeded in a 24 well plate with 500 µl cell suspension per well. For protein extraction and western blotting, cells were seeded in 10 cm cell culture dishes. 24 hours after seeding the cells were treated with the indicated concentrations of BV6 and IFNα.

2.3 Cell death assays

2.3.1 Determination of cell viability via MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is a versatile and easy to use colometric assay to quantify cell metabolic activity *in vitro*

reflecting cell viability.[273] Cellular oxidoreductase enzymes of viable cells' mitochondria can reduce the bright yellow tetrazolium salt MTT to insoluble, purple formazan. The photometric absorbance of formazan can be measured via spectrophotometry. The amount of light absorbance is proportional to the concentration of formazan and hence to the amount of viable cells in the dilution. Cells were seeded in 96 well plates 24 hours prior to the treatment. Cells were treated as indicated and the medium was replaced by 10 µl of MTT solution (5 mg MTT diluted in 1 ml of sterile PBS). Cells were incubated for 3 hours at 37° C and the formazan was resuspended in 100 µl isopropanol per well. Absorbance was measured at 550 nm using a spectrophotometer.

2.3.2 Determination of DNA fragmentation by flow cytometry

Activation of cellular endonucleases and subsequent DNA fragmentation is a hallmark of apoptotic cell death and can be quantified by analyzing propidium iodide (PI)-stained cells.[274] PI is a red-fluorescent agent that can bind to DNA by intercalating between the nucleobases. After binding, its fluorescence is enhanced and the fluorescence maximum is shifted 30-40 nm to red. The intensity of the fluorescence correlates with the amount of DNA the stained cells contain. Since endonucleases degrade DNA, nuclei of apoptotic cells contain less DNA than normal cells in G_o or G_1 phase (hypoploic, sub-G1-cells). The sub-G1-peak can be used to determine the relative amount of apoptotic cells in the population. Cells were seeded in 24 well plates 24 hours prior to treatment. Cells were treated as indicated and the supernatant was removed. The cells were detached by adding 200 µl Trypsin and incubation at 37° C for 5 minutes. Cells were washed with ice-cold PBST and centrifuged twice (1800 rpm, 5 minutes, 4° C). Since PI is a polar agent and cannot permeate the cellular membrane, cells were incubated in a hypotonic fluochrome buffer (50 µg/ml PI, 0.1% sodium citrate and 0.1% Triton-X 100) for at least 1 hour at 4° C protected from light to achieve membrane permeabilization and DNA binding of PI. The relative amount of hypoploic sub-G1-cells was determined by flow cytometry in the PE channel at 488 nm.

2.3.3 Determination of membrane permeabilization by flow cytometry

The constriction and permeabilization of the cellular membrane is another hallmark and morphological characteristic of apoptotic cells and its assessment can be used as an additional assay to detect apoptotic cells and to verify the results of DNA fragmentation assays. Membrane pemeabilization was assessed by staining cells with the red-fluorescent intercalating agent PI. To achieve this, cells were seeded at 24 well plates 24 hours prior to treatment. Cells were then treated as indicated and the supernatant was removed. The cells were detached by adding 200 µl Trypsine and incubating at 37° C for 5 minutes. Cells were washed with ice-cold PBST and centrifuged twice (1800 rpm, 5 minutes, 4° C). Since the cells were not incubated in a hypotonic buffer to induce membrane permeabilization, PI can only permit into cells with disintegrated cellular membranes. Immediately before PI positivity was measured by flow cytometry, cells were treated with 10µl of PI (final concentration 1 µg/ml, diluted in PBS).

2.3.4 NFκB reporter assay

The NFκB reporter assay is an assay for monitoring the activity of NFκB-regulated signaling pathways *in vitro*. The cells were transduced with an expression vector encoding the enhanced green fluorescent protein (eGFP) under the control of an NFκB consensus transcriptional response element (vector pTRH1 NF-κB-EGFP). Induction of canonical as well as non-canonical NFκB signaling results in the nuclear binding of the RelA:p50 or RelB:p52 dimers to the response element subsequently enhancing the eGFP-expression. eGFP is a genetically engineered mutant of wild-type green fluorescent protein (GFP) that matches the spectral characteristics of flow cytometry filter sets and can be measured at 488 nm. Since eGFP is a relatively small and biologically inert protein the increase in eGFP-fluorescence determined by flow cytometry analysis of median FITC intensity correlates with the amount of NFκB signaling *in vitro*. Cell culture of the reporter cells was done analog to the A172 wild-type cells.

2.4 Protein analysis and statistical methods

2.4.1 Cell lysis and protein extraction

In order to determine protein expression levels, cell lysis and protein extraction must be performed prior to western blot analysis. Cells were seeded in 10 cm dishes 24 hours prior to treatment. Cells were then treated as indicated. The treated cells were scraped off the dishes and transferred into a Falcon tube using PBS. The suspension was centrifuged (1800 rpm, 5 minutes, 4° C) and the cell pellet was resuspended in lysis buffer and incubated on ice for 25 minutes. The solution was then centrifuged (14000 rpm, 20 minutes, 4° C), supernatant was transferred to a fresh tube and protein concentration was determined using the Pierce™ BCA Protein Assay Kit and the Sunrise™ Tecan according to the manufacturer's instruction.

2.4.2 SDS polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a commonly used method for the analytical separation of proteins based on their molecular size. It is based on the fact that charged molecules such as proteins migrate in an applied electric field towards an electrode of the opposite sign. In its native state, the velocity of the molecule depends on both its size and charge as well as the magnitude of the applied electric field. In order to separate molecules by molecular weight only, the proteins tertiary structure needs to be denaturated and its intrinsic molecular charge needs to be masked uniformly. This can be achieved by adding SDS to the protein dilution. SDS is a negatively charged detergent that uniformly coats and unfolds proteins resulting in linear, SDS-coated proteins with an even charge to mass ratio. Hence electrophilic migration towards the positively charged anode only depends on the protein's molecular mass. The gel matrix used for the SDS-PAGE consists of polyacrylamide, a chemically inert polymer that can be produced in variant pore sizes to match different separation conditions.

Each sample contained 50 µg of protein and was denatured in SDS-loading buffer for 5 minutes at 96 °C. The gel (5% polyacrylamide for stacking gel, 12% polyacrylamide for

separation gel) was placed in the running tank filled with running buffer and the samples were delivered to the wells. PageRuler Plus prestained protein ladder was loaded on the first and the last well of the gel. The electrophoresis run at constant voltage until the dye front reached the bottom end of the gel (100 V for the first 30 minutes followed by 140 V until the end).

2.4.3 Western blot

After separation in gel electrophoresis, the proteins are transferred to a membrane and stained with specific antibodies. This analytic process is called western blot analysis. Similar to gel electrophoresis, an electric field, applied perpendicular to the polyacrylamide membrane is used to transfer the SDS-coated proteins to a nitrocellulose membrane. For this, the gel was blotted at a constant amperage of 1 mA/cm² for 90 minutes. Then, the membrane was washed with PBST for 10 minutes and put in blocking buffer for 1 hour in order to avoid unspecific binding of antibodies to the membrane. The membrane was then washed three times with PBST and the primary antibody was added to the membrane and incubated overnight at 4° C. The membrane was then washed three times with PBST. The second antibody was added and incubated for one hour at room temperature. The membrane was again washed three times with PBST. Two different approaches were used for the detection of the primary antibody. Enhanced chemiluminescent detection (ECL) uses secondary antibodies conjugated with HRP that catalyze the light-emitting oxidation of luminol. Pierce® ECL western blotting detection reagent along with developer and fixing solution was used to detect the chemiluminescence and subsequently the target protein on an ECL hyperfilm. Alternatively, fluorescently labeled secondary antibodies were directly visualized by using the infrared Odyssey® imaging system.

2.4.4 Statistical analysis

Statistical significance was determined using Student’s t-test (two-tailed distribution, two sample, unequal variance) and was defined as significant at $p<0.05$ (*) and as highly

significant at $p<0.01$ (**) and $p<0.001$ (***). The drug interaction was analyzed based on the method described by Chou [275,276] using the CalcuSyn software (Biosoft, Cambridge, UK). A combination index (CI) <0.9 indicates synergism, CI 0.9-1.1 additivity and >1.1 antagonism.

3 RESULTS

3.1 BV6 and IFNα as single treatment

3.1.1 BV6 induces cell death in GBM cells in a dose-dependent manner

First we investigated the potential of single compound BV6 and IFNα to induce cell death in GBM cells to identify subtoxic concentrations. The aim was to evaluate the BV6 and IFN-induced loss of cell viability and to identify suitable cell lines for combination testing. Five GBM cell lines were cultivated and treated with increasing BV6 concentrations and cell death was assessed using a MTT cell viability assay after 72 hours. The initial dose ranges were chosen based on preliminary experiments.

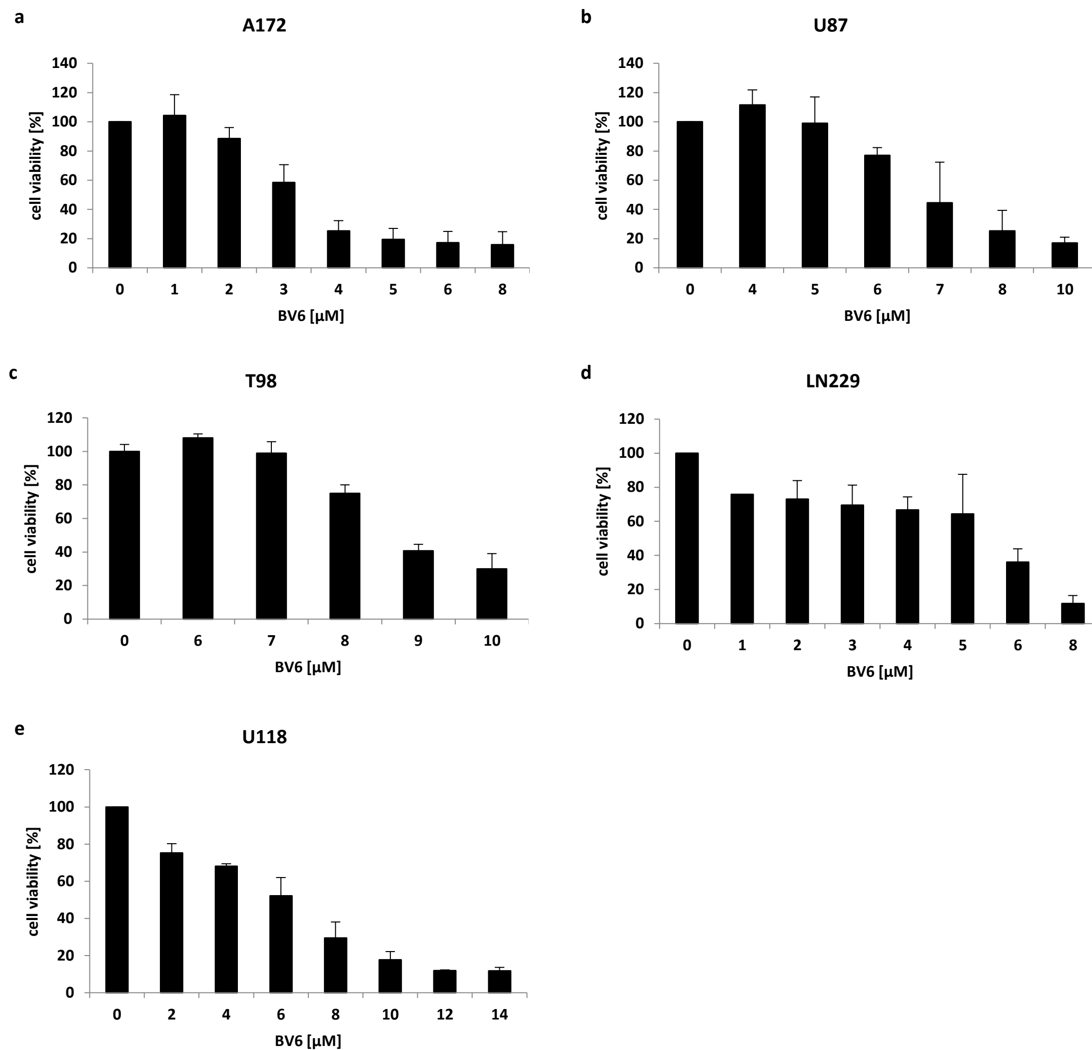

Figure 7 BV6 induces loss of cell viability in a dose-dependent manner.

Five different glioblastoma cell lines were treated for 72 hours with the indicated BV6 concentrations and the cell viability was measured by MTT staining assay. The results are displayed as percentage of the untreated cells. Mean ± S.D. of at least three independent experiments performed in triplicates are shown.

BV6 induces loss of cell viability in several GBM cell lines in a dose-dependent manner (Fig. 7). Quantification of cell death was achieved by measuring the metabolic activity of the treated cells and comparing it with an untreated control population. Since this method does not detect cell death in particular, no further conclusions can be made whether the decrease in metabolic activity results in the cells actually dying or is due to

alternative mechanism, e.g. proliferation arrest. Nor can it distinguish between different modes of cell death such as apoptosis or necrosis. Observation of the treated cells with the light microscope revealed morphological changes characteristic for regulated cell death, in particular cell detachment from the surface and formation of membrane blebs. From the treated cell lines, A172 cells show a marked decrease in cell viability upon treatment with 3 µM BV6. The initial increase of cell viability observed in A172, U87 and T98 cells may be due to an NFκB-dependent tumorigenic effect of non-toxic BV6 concentrations that has been described earlier for GBM cells.[277,278]

U87 and U118 cells show similar sensitivity to BV6 single treatment, although higher concentrations are necessary to achieve equal amount of cell viability reduction. In contrast, T98 and LN229 show only little response to the BV6 single treatment.

Taken together, BV6 single treatment markedly reduced cell viability in A172, U87 and U118 cells in low concentrations while higher levels of BV6 were necessary for the same reduction in T98 and LN229 cells.

3.1.2 IFNα single treatment reduces cell viability in GBM cells in a dose-dependent manner

To observe whether IFNα single treatment reduces cell viability, we treated GBM cell lines for 72 hours with increasing concentrations of IFNα and assessed cell viability by MTT staining. On the basis of the currently available evidence, we expected IFNα single treatment to slightly reduce cell viability since it has been reported to induce apoptosis in several cell lines. However, since IFNα is a key negative regulator of cell-cycle progression, we assumed cell-cycle arrest to be the main cause for reduced viability after IFNα single treatment.

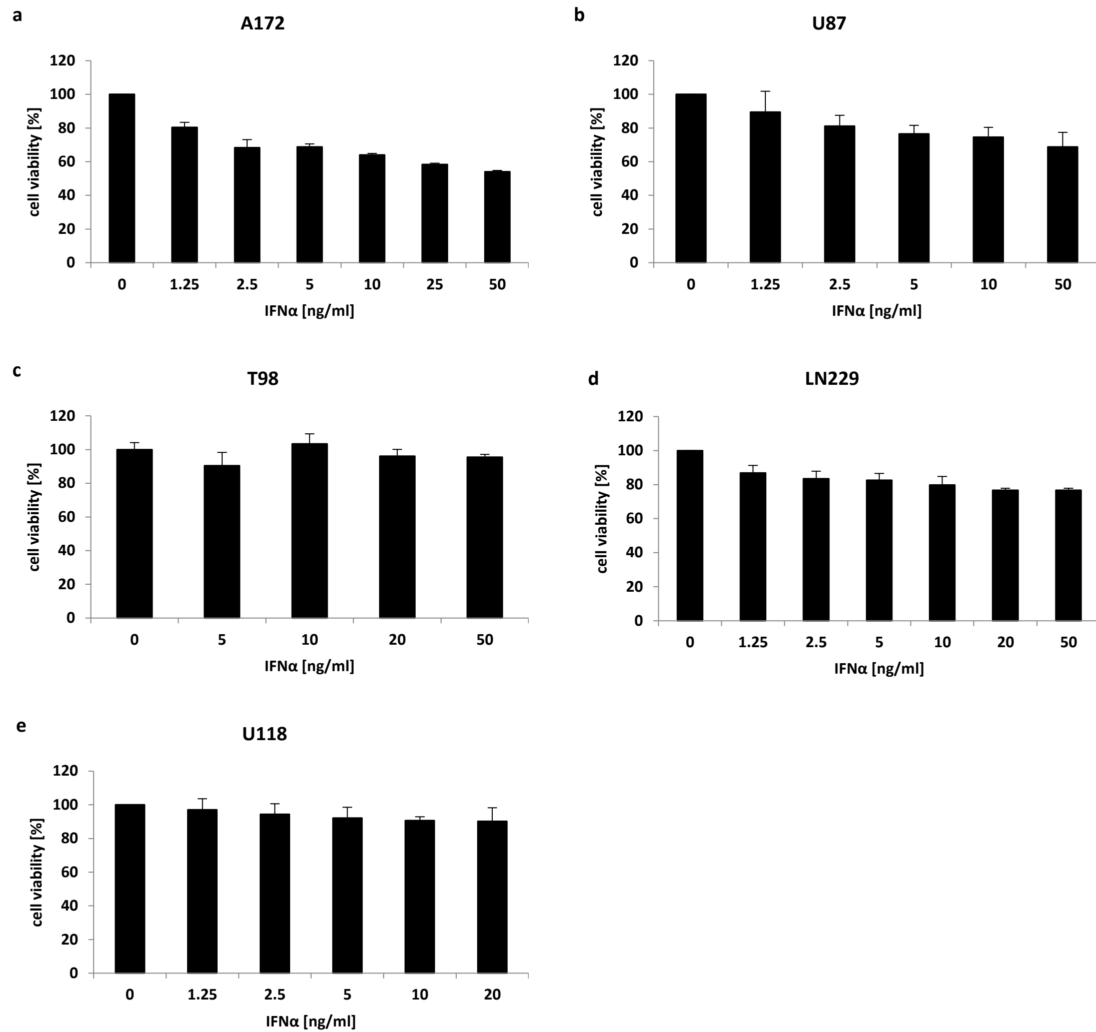

Figure 8 IFNα induces loss of cell viability in a dose-dependent manner.

Five different glioblastoma cell lines were treated for 72 hours with the indicated IFNα concentrations and the cell viability was measured by MTT staining. The results are displayed as percentage of the untreated cells. Mean ± S.D. of at least three independent experiments performed in triplicates are shown.

The IFNα-dependent reduction of cell death in GBM cells was minor compared to BV6 single treatment. The extent of cell viability reduction was strongest for A172 cells, considerably for U87 and LN229 cells and almost absent for T98 and U118 cells (Fig. 8).

3.1.3 BV6 and IFNα induce DNA fragmentation in GBM cell lines

To corroborate the cell death induction by BV6 and IFNα single treatment in GBM cell lines, we assesed DNA fragmentation, one of the molecular hallmarks of apoptotic cell death. To this end, we focused our experiments on the most reponsive cell lines, namely A172, U87 and T98 cells.

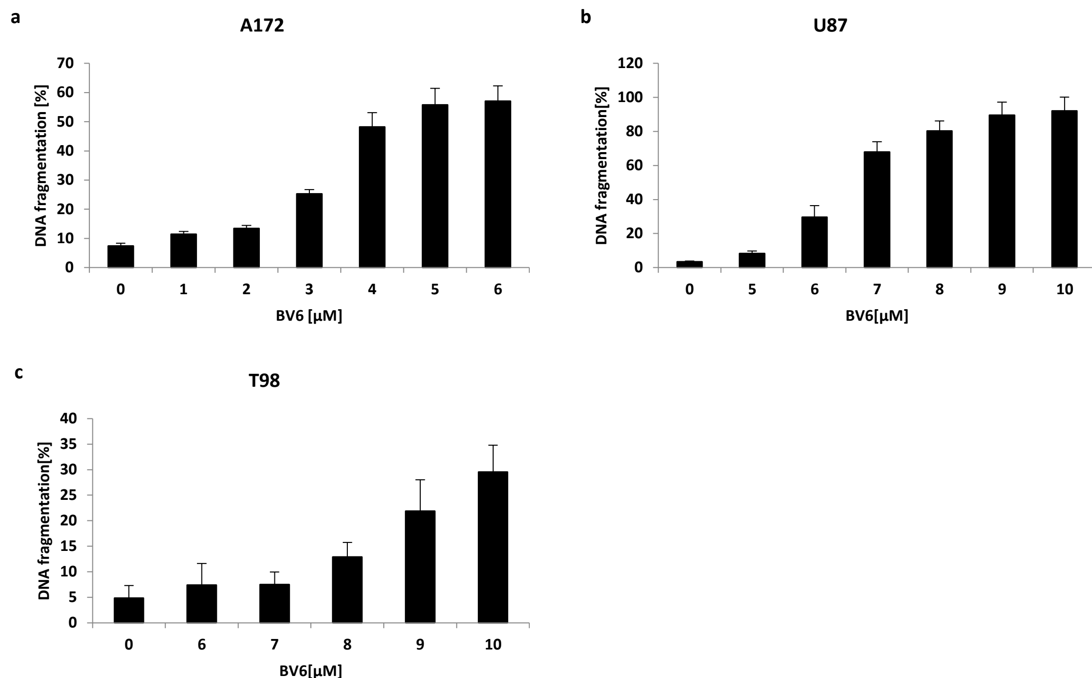

Figure 9 BV6 induces DNA fragmentation in a dose-dependent manner

The cells were treated for 72 hours with the indicated concentrations and stained with Nicoletti buffer. The DNA fragmentation was measured by flow cytometry and expressed as the percentage of the untreated cells. Mean ± S.D. of at least three independent experiments performed in duplicates are shown.

BV6 notably induces DNA fragmentation in a dose-dependent manner. The graph shows a steep increase for A172 cells between 4µM and 5µM BV6 (Fig. 9 a). Similarly, DNA fragmentation of U87 cells sharply increases from 6 µM to 7 µM BV6 (Fig. 9 b). T98 cells show a slow increase in DNA fragmentation and remain stable at roughly 30% compared to the control population.

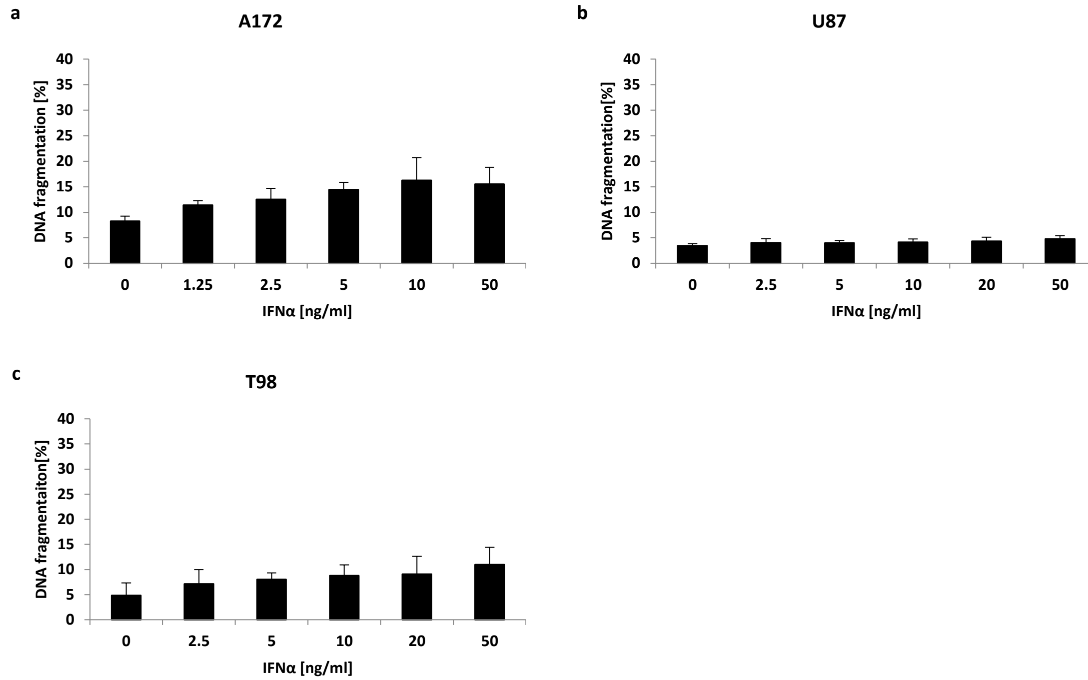

Figure 10 IFNα induces DNA fragmentation in a dose-dependent manner.

The cells were treated for 72 hours with the indicated concentrations of IFNα and stained with Nicoletti buffer. The DNA fragmentation was measured by flow cytometry and expressed as percentage of the untreated cells. Mean ± S.D. of at least three independent experiments performed in duplicates are shown.

IFNα, when administered as a single agent, scarcely induced DNA fragmentation. This supports the assertion that IFNα single treatment may have salient anti-proliferative but negligibly pro-apoptotic effects in GBM cells.

3.2 BV6 and IFNα as combination treatment

3.2.1 BV6 and IFNα cooperate to induce loss of cell viability in GBM cells

To explore whether BV6 and IFNα act in concert to induce cell death in GBM cells, we treated A172, U87 and T98 cells with increasing subtoxic concentrations of BV6 and IFNα for 72 hours and assessed the reduction of cell viability by MTT staining (Fig. 11).

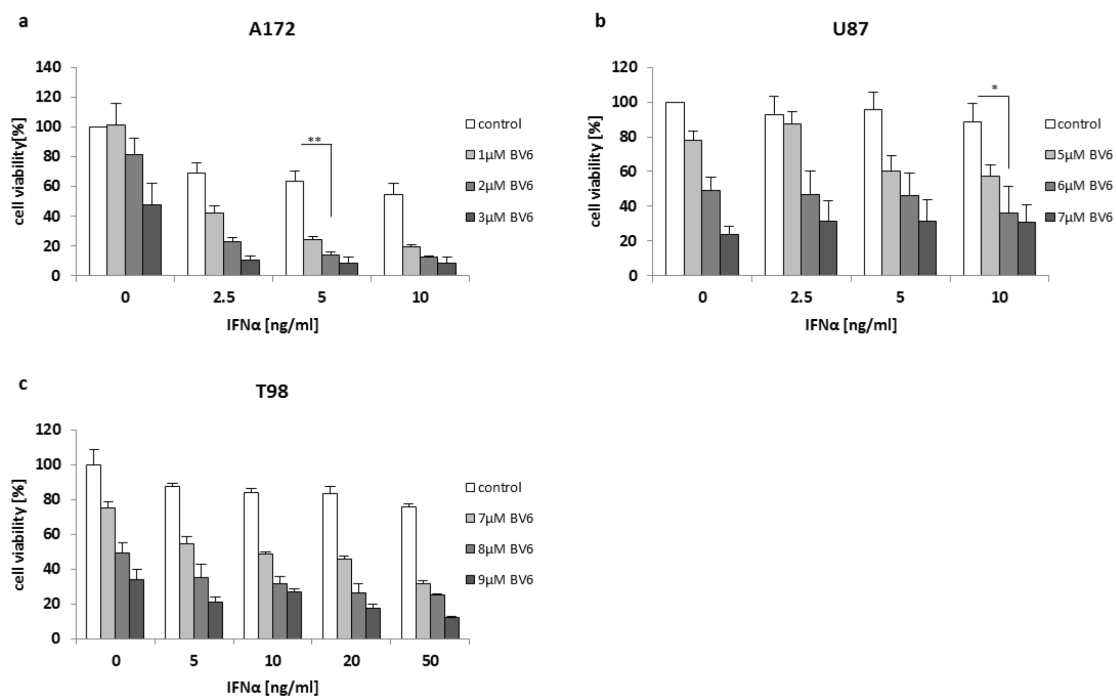

Figure 11 The combination of BV6 and IFNα induces loss of cell viability in a dose-dependent manner

The cells were treated for 72 hours with the indicated combinations of BV6 and IFNα. The cell viability was measured by MTT and expressed as percentage of the untreated cells. Mean ± S.D. of at least three independent experiments performed in triplicates are shown. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

In A172 cells, combination of BV6 and IFNα markedly reduced cell viability over a course of 72 hours. The combination of 2 µM BV6 and 5 ng/ml IFNα significantly reduced cell viability compared to either treatment alone (Fig. 11 a). Furthermore, low

concentrations of either compound were sufficient to achieve a considerable amount of cell death. In U87 cells the combination of 5 µM BV6 and 10 ng/ml IFNα significantly reduced the cell viability compared to the single treatments (Fig 11 b). In T98 cells, BV6 and IFNα combination treatment slightly induced loss of cell viability compared to either single treatment which was not statistically significant (Fig. 11 c). As a consequence, T98 cells were excluded from further experiments.

3.2.2 BV6 and IFNα cooperate to induce DNA fragmentation in GBM cells

To corroborate the MTT assays results by another cell death assay, we again assessed DNA fragmentation in A172 and U87 cells. Additionally, we monitored the kinetics of the treatment over a time period up to 72 hours for A172 cells. Furthermore, we assessed BV6-dependent degradation of IAPs, namely cIAP1, cIAP2 and XIAP.

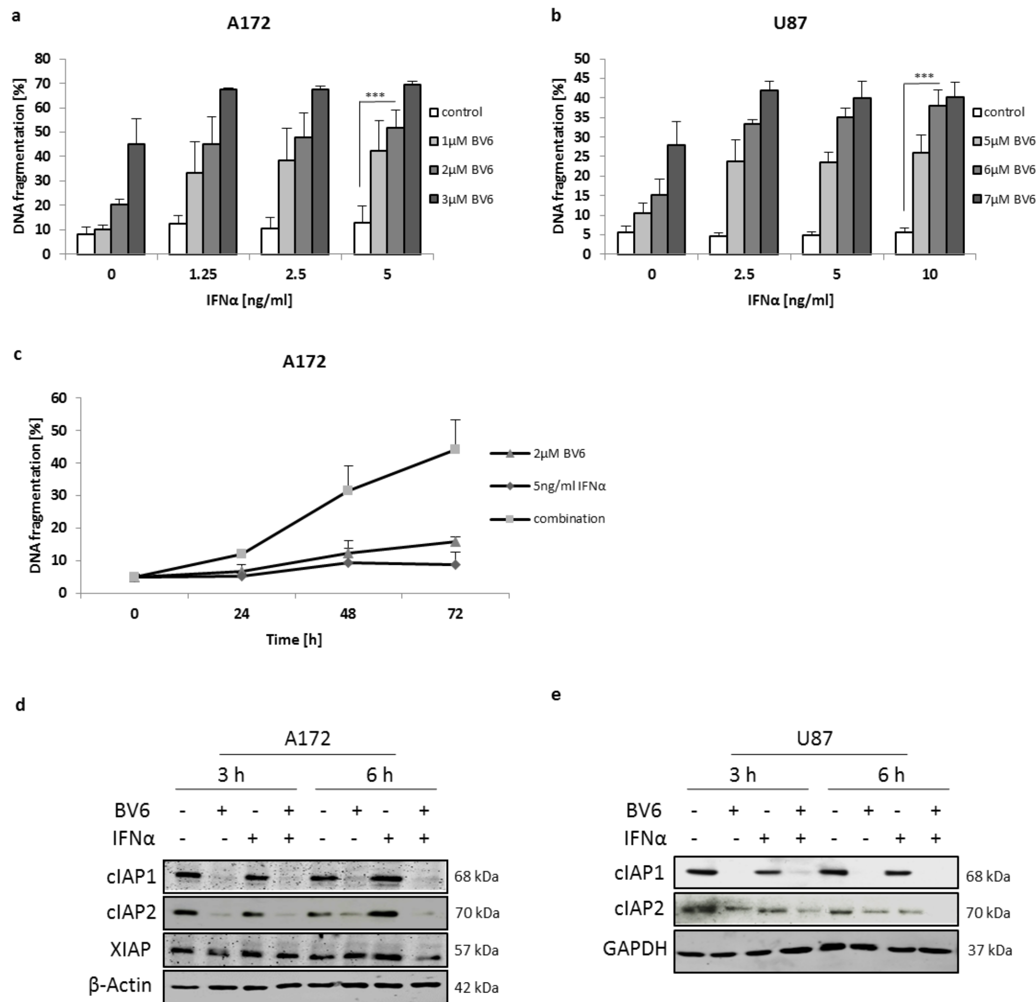

Figure 12 The combination of BV6 and IFNα induces DNA fragmentation in glioblastoma cells in a dose-dependent manner

The cells were treated for 72 hours with the indicated combination of BV6 and IFNα and stained with Nicoletti buffer. The DNA fragmentation was measured by flow cytometry and expressed as percentage of the untreated control cells. All experiments are n = 3 and the mean ± S.D. is shown. (**c**) The cells were treated for the indicated time points with a combination of 2 µM BV6 and 5 ng/ml IFNα and stained with Nicoletti buffer. The DNA-fragmentation was measured by flow cytometry. Mean ± S.D. of at least three independent experiments performed in duplicates are shown. *, p < 0.05; **, p < 0.01; ***, p < 0.001. (**d** and **e**) BV6 and IFNα cooperate to trigger depletion of IAP proteins in A172 and U87 glioblastoma cells. The cells were treated for 3 hours or 6 hours with a combination of BV6 and IFNα (2 µM BV6 + 5 ng/ml IFNα for A172, 6 µM BV6 and 10 ng/ml IFNα for U87). Protein levels were assessed by western blotting and GAPDH and β-Actin were used as loading control.

The BV6/IFNα-cotreatment significantly increased the DNA fragmentation in A172 (2 µM BV6 + 5 ng/ml IFNα, $p<0.001$) and U87 (6 µM BV6 + 10 ng/ml IFNα, $p<0.001$) cells (Fig. 12 a and b). Monitoring the induction of cell death revealed a steady growth over the period of 72 hours. Hence, BV6 and IFNα cooperate to induce cell death in a time- and dose-dependent manner in GBM cells (Fig. 12c).

BV6/IFNα-cotreatment rapidly decreased cIAP1 and cIAP2 expression levels in A172 and U87 cells (Fig. 12 d and e). In contrast, the cotreatment did not notably interfere with XIAP protein levels, consistent with previous data showing that Smac mimetics induce proteasomal degradation of cIAP1/2 but not XIAP.[147] However, Smac mimetics bind BIR2 and BIR3 motifs of XIAP via their AVPI-stretch and disrupt XIAP-caspase interaction.[137]

3.2.3 BV6 and IFNα cooperate to induce membrane permeabilization in GBM cells

To verify the cooperative effect of IFNα and BV6 and to validate our previous results with a second cell death assay we assessed cell membrane permeabilization using flow cytometry on PI-stained cells. Again, we monitored the kinetics of the treatment over a course of 72 hours.

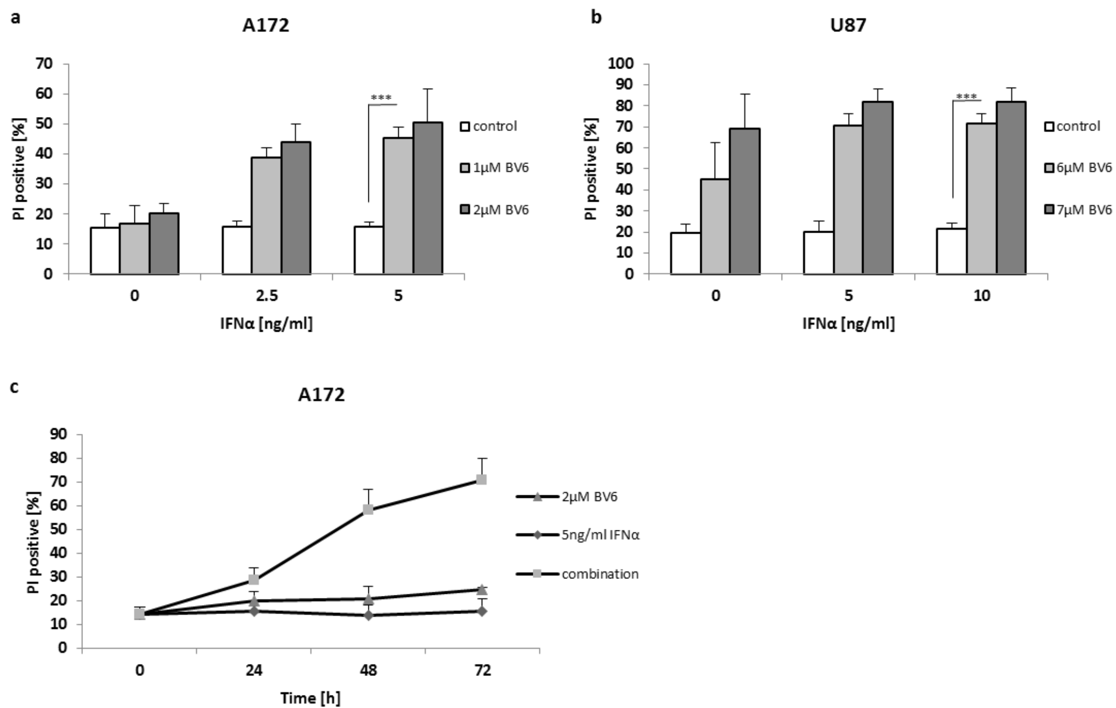

Figure 13 The combination of BV6 and IFNα induces permeabilization of the cell membrane in glioblastoma cells in a dose-dependent manner.

The cells were treated for 72 hours with the indicated combination of BV6 and IFNα and stained with PI. The PI positive cells were measured by flow cytometry and expressed as percentage of the untreated cells. All experiments are n = 3 and the mean ± S.D is shown. (**c**) The permeabilization of the membrane increases over the course of 72 hours in A172 glioblastoma cells. The cells were treated for the indicated time points with the combination of 2µM BV6 and 5ng/ml IFNα and stained with PI. The percentage of PI positive cells was measured with flow cytometry. Mean ± S.D. of at least three independent experiments performed in duplicates are shown. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

The combinations of BV6 and IFNα significantly induced membrane permeabilization in A172 (2 µM BV6 + 5 ng/ml IFNα, $p<0.001$) and U87 (6 µM BV6 + 10 ng/ml IFNα, $p<0.001$) cells. Comparably to DNA fragmentation, the effect increased steadily over the period of 72 hours in A172 cells. Thus, the data gathered support the assumption that BV6 and IFNα cooperate to induce cell death in GBM cells.

3.2.4 Synergistic interaction of BV6 and IFNα is confirmed by CI calculation

To confirm the synergistic interaction of BV6 and IFNα we calculated the combination index (CI) for each combination based on the results of DNA fragmentation analysis by flow cytometry (Fig. 12). CI < 0.9 indicates synergism, 0.9 – 1.1 additivity and CI > 1.1 indicates antagonism.

CI values for A172

BV6[µM] IFNα [ng/ml]	1	2	3
1.25	0.398	0.604	0.531
2.5	0.351	0.566	0.530
5	0.321	0.515	0.503

CI values for U87

BV6[µM] IFNα [ng/ml]	5	6	7
2.5	0.736	0.783	0.828
5	0.742	0.767	0.846
10	0.715	0.741	0.846

Table 1 CI calculation for the BV6/IFNα cotreatment in A172 and U87 based on the results of DNA fragmentation.

CI <0.9 indicates synergism, CI 0.9-1.1 additivity and CI > 1.1 indicates antagonism.

CI calculation confirmed that the interaction of BV6 and IFNα is synergistic. For further understanding of the molecular mechanisms underlying the cotreatment, we focused on the combination of 2 µM BV6 and 5 ng/ml IFNα in A172 cells (CI value 0.515, p<0.001 in Fig. 12) and 5 µM BV6 and 10 ng/ml IFNα in U87 cells (CI value 0.741, p<0.001 in Fig. 12). These combinations have on the one hand a low cytotoxicity as single compounds. On the other hand, as combination treatment they induce cell death in a synergistic manner.

3.3 Molecular mechanism of cell death induction by BV6/IFNα

3.3.1 Inhibition of caspases effectively impairs induction of cell death by BV6/IFNα-cotreatment

Previous publications suggest a TNFα-dependent activation of procaspase-8 and subsequent induction of apoptosis as a pivotal molecular mechanism for antitumoral activity and cytotoxicity of Smac mimetics. We hypothesized that pharmacological inhibition of caspase activation would impair BV6/IFNα-induced reduction of cell viability and DNA fragmentation. Therefore, we used the small molecule pan-caspase inhibitor zVAD.fmk to block caspase activation in BV6/IFNα-cotreated cells. Moreover, we used western blotting to detect caspase cleavage products in cells treated with either BV6 and IFNα and the combination of both to confirm caspase activation.

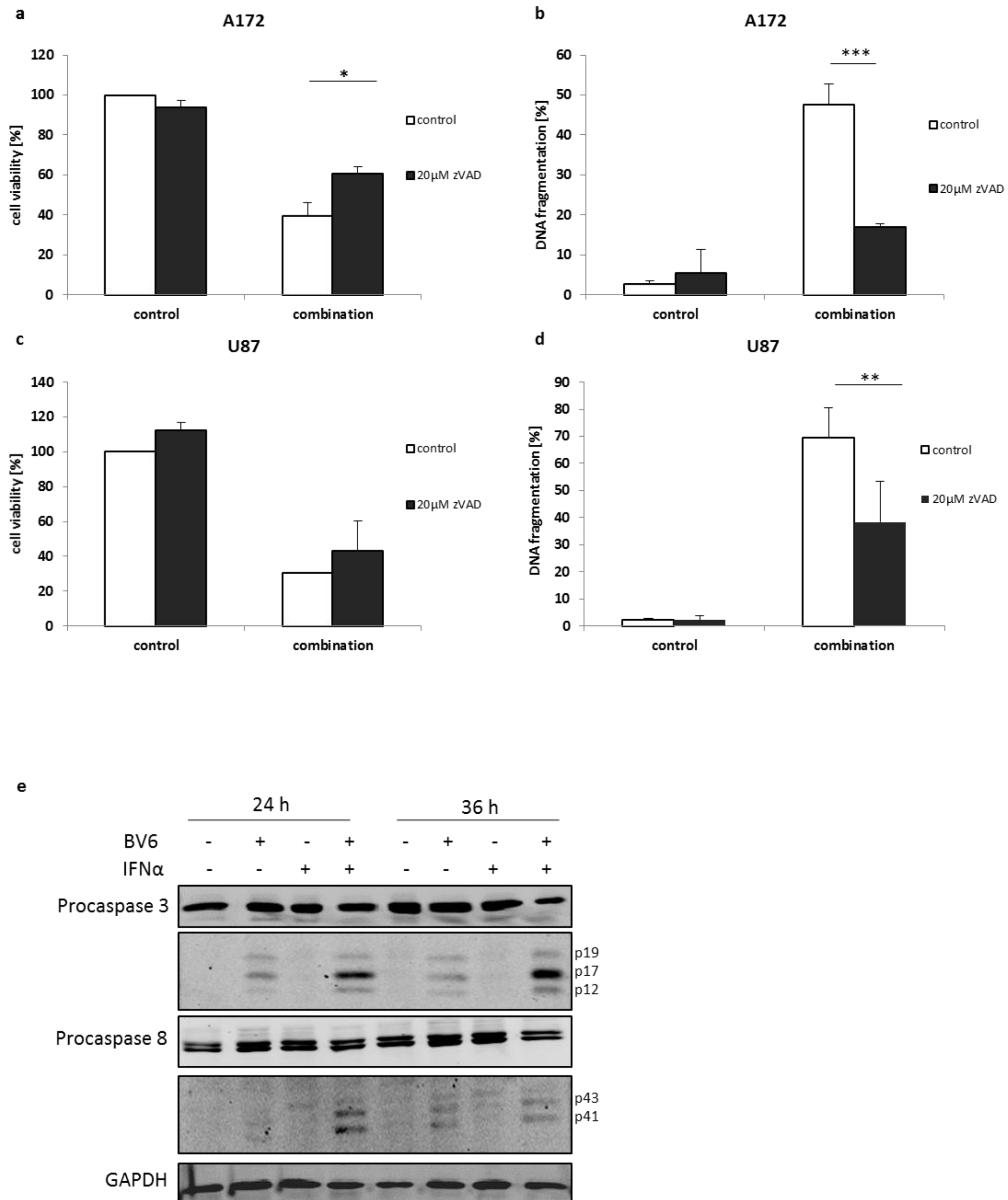

Figure 14 Caspases play an important role in the induction of loss of cell viability and DNA fragmentation induced by the combination of BV6 and IFNα

(**a** and **c**) Cells were treated for 72 hours with the combination of BV6 and IFNα (2 µM BV6 + 5 ng/ml IFNα for A172, 6 µM BV6 and 10 ng/ml IFNα for U87) with or without 20 µM of the pan-caspase inhibitor zVAD.fmk. Loss of cell viability was measured by MTT staining. (**b** and **d**). DNA fragmentation was measured by flow cytometry after staining the cells with Nicoletti buffer. Mean ± S.D. of at least three

independent experiments performed in triplicates (MTT) or duplicates (DNA fragmentation) are shown. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. (**e**) A172 glioblastoma cells were treated for the indicated time points with the combination of 2 µm BV6 and 5 ng/ml IFNα and protein levels for procaspase 3 and 8 and their cleavage products were assessed by western blotting. GAPDH was used as loading control.

Consistent with the established molecular model for Smac mimetic-induced cell death, inhibition of caspase activation significantly reduced loss of cell viability and DNA fragmentation in A172 cells (Fig. 14 a and b). In U87 cells, inhibition of caspase activation significantly decreased BV6/IFNα-induced DNA fragmentation (Fig. 14 d). In addition, MTT staining revealed a slight but not statistically significant rescue from loss of cell viability in zVAD.fmk-treated U87 cells (Fig. 14 c).

Detection of procaspase-3 and -8 cleavage fragments in A172 cells by western blot confirmed caspase activation in BV6 single treated and BV6/IFNα-cotreated cells after 24 hours and 36 hours (Fig. 14 e). Of note, caspase-8 and -3 activation was more increased in cotreated cells compared to BV6 single treatment, indicated by increased accumulation of cleavage products and decreased pro-enzyme levels. Procaspase-8 activation peaked after 24 hours and thereafter declined with minor activation still present after 36 hours. Caspase-3 cleavage products slightly increased between 24 hours and 36 hours.

3.3.2 BV6/IFNα-cotreatment induces activation of NFκB signaling

It is a widely held view that Smac mimetic harness NFκB-dependent TNFα expression to induce cell death in treated cells. Consistently, IAP inhibitor-induced apoptosis was shown to require unimpaired NFκB signaling.[279] Since we assumed a similar mechanism for BV6/IFNα-cotreatment, we examined the effects of BV6/IFNα-cotreatment on NFκB activation and asked whether attenuated activation of NFκB TFs reduced cell death in GBM cells.

To monitor activation of the canonical NFκB pathway, we treated A172 and U87 cells with BV6 and IFNα single and cotreatment and detected expression levels of key proteins after 3 and 6 hours post treatment using western blotting.

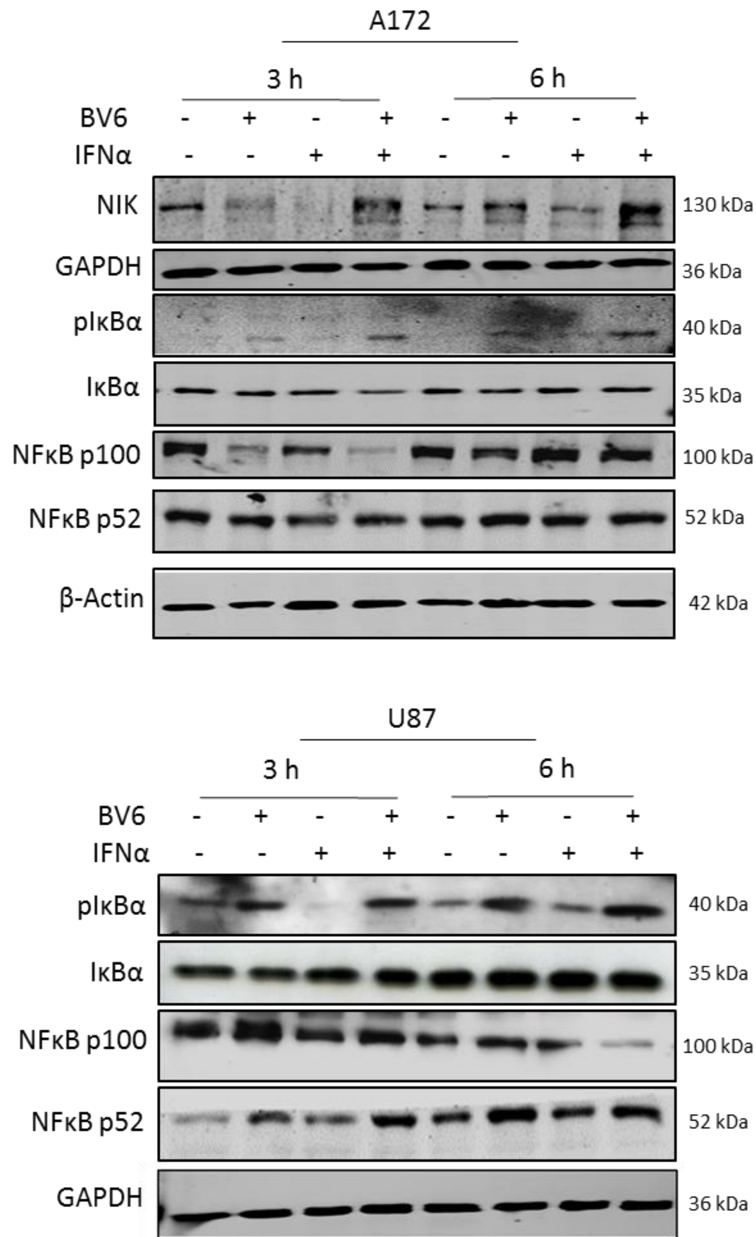

Figure 15 BV6 and IFNα cooperate to trigger depletion of IAP proteins and activate key proteins of the NFκB pathway in A172 and U87 glioblastoma cells

A172 cells were treated for 3 or 6 hours with a combination of BV6 and IFNα (2 µM BV6 + 5 ng/ml IFNα for A172, 6 µM BV6 and 10 ng/ml IFNα for U87). Protein levels were assessed by western blotting and GAPDH and β-Actin were used as loading control.

BV6/IFNα cotreatment rapidly increased NIK levels in A172 cells and induced phosphorylation of IκBα in A172 and U87 cells accompanied by proteasomal processing

of p100 (Fig. 15). NIK, a critical upstream protein of the canonical NFκB pathway, is negatively regulated by cIAP1 and cIAP2 and BV6-dependent depletion of both IAPs may account for NIK stabilization and subsequent phosphorylation of IκBα and proteasomal processing of p100 to its mature form p52. By comparison, BV6/IFNα cotreatment-induced NIK accumulation and subsequent canonical NFκB signaling substantially exceeded that of BV6 single treatment. Interestingly, IFNα single treatment slightly increased NIK levels after 6 hours but did not affect IκBα, pIκBα or p100 protein levels.

3.3.3 Inhibition of NFκB activation rescues cells from BV6/IFNα-induced cell death

Next, we asked whether unimpaired NFκB signaling is necessary for BV6/IFNα-cotreatment-induced cell death in GBM cells. To this end, we established A172 and U87 cells that stably express a dominant negative mutant of IκBα that is not susceptible to phosphorylation and subsequent proteasomal degradation (IκBα-superrepressor (IκBα-SR), IκBα-empty vector (IκBα-EV)). IκBα-EV and IκBα-SR cells were kindly provided by I. Eckhardt.

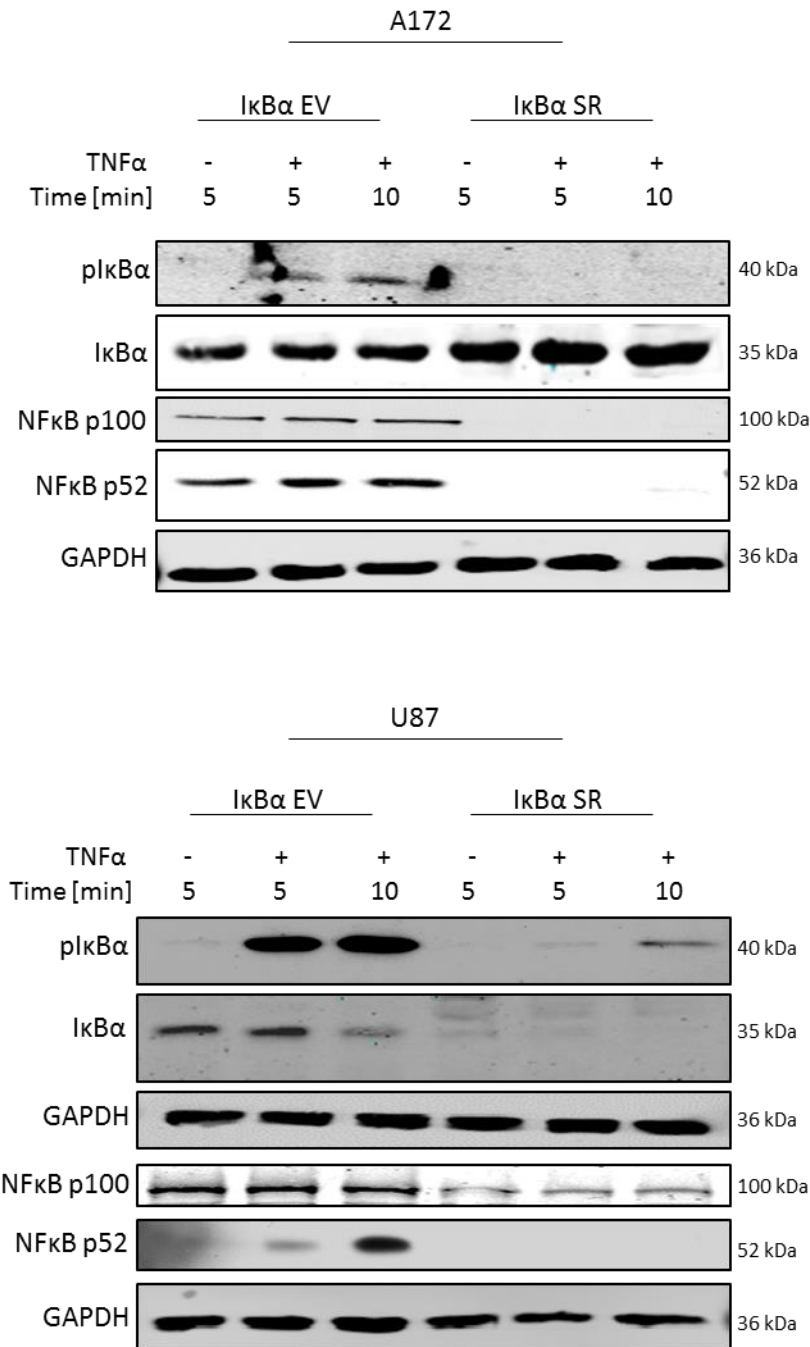

Figure 16 In A172 and U87 IκBα-SR cells, TNFα fails to induce phosphorylation of IκBα and subsequent activation of the NFκB signaling pathway

Cells were treated for 5 and 10 minutes with 5 ng/ml TNFα. Protein levels were assessed by western blotting and GAPDH was used as a loading control.

First we investigated whether IκBα-SR expressing cells showed impaired NFκB signaling. To this end we treated IκBα cells with the prototypical NFκB stimulus TNFα and assessed NFκB activation using western blotting (Fig. 16). In A172 and U87 IκBα-EV cells, TNFα

rapidly induced phosphorylation of IκBα to its phosphorylated isoform pIκBα and expression of NFκB target genes IκBα, p100 and p52. In A172 and U87 IκBα-SR cells, TNFα failed to induce phosphorylation of IκBα and the expression of the NFκB target genes IκBα, p100 to p52. Moreover, in untreated IκBα-SR cells, basal levels of p100 are markedly reduced compared to IκBα-EV cells which plays an additional role in the inhibition of non-canonical NFκB signaling. Notably, in U87 IκBα-SR cells, a slight increase in protein levels of the phosphorylated NFκB target gene IκBα was detectable after 10 minutes of TNFα treatment, indicating submaximal inhibition of NFκB signaling in U87 and methodical limitations of this cell model. Taken together these data suggest that the IκBα-SR suppresses both the canonical and non-canonical NFκB pathway.

Next, we treated IκBα-EV and IκBα-SR cells with BV6 and IFNα in single and cotreatment and cell death was assessed by MTT assay and flow cytometry. Reduction of cell viability and induction of DNA fragmentation in IκBα-SR cells were compared with empty vector control cells

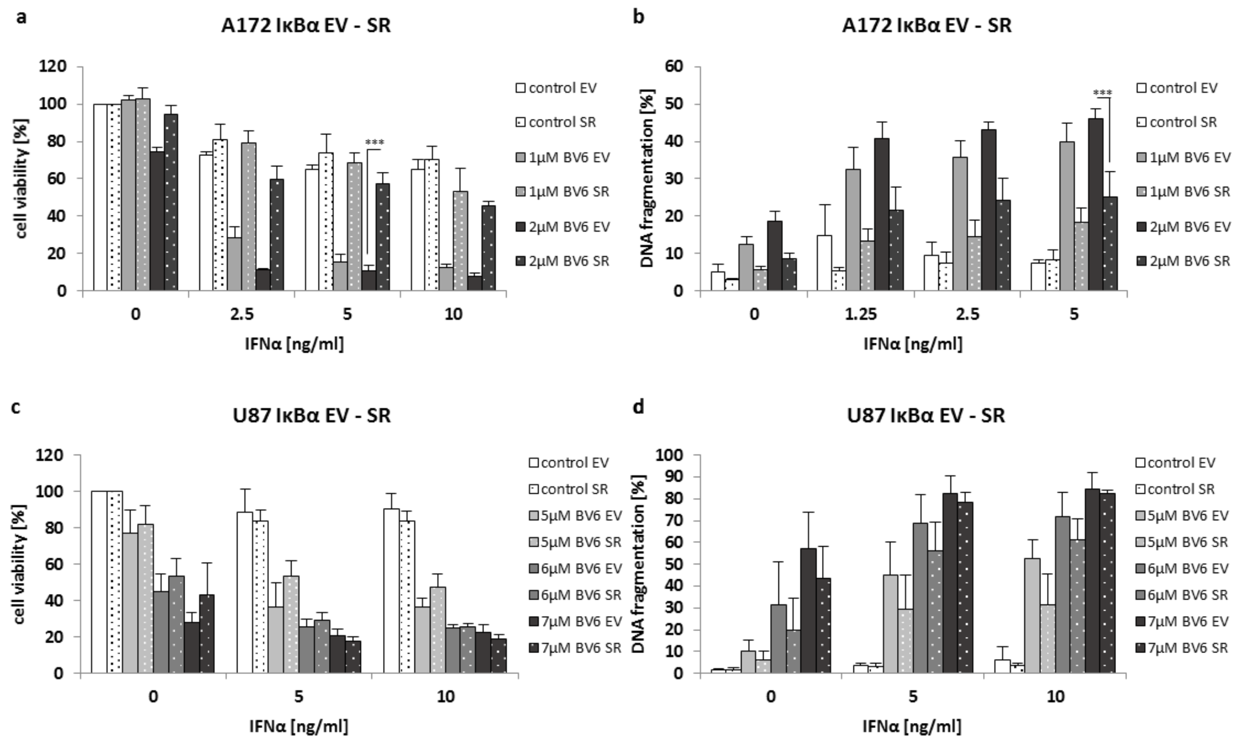

Figure 17 Inhibition of the canonical NFκB pathway diminishes the loss of cell viability and the DNA fragmentation that is induced by the combination of BV6 and IFNα

A172 and U87, IκBα each empty vector (EV) and IκBα super repressor (SR), were treated for 72 hours with the indicated concentrations of BV6 and IFNα. (**a** and **c**) Cell viability was measured by MTT staining. (**b** and **d**) DNA fragmentation was measured by flow cytometry after staining the cells with Nicoletti buffer. Mean ± S.D. of at least three independent experiments performed in triplicates (MTT) or duplicates (DNA fragmentation) are shown. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

In A172 cells, inhibition of IκBα-dependent NFκB activation significantly rescued cells from BV6/IFNα-induced loss of cell viability (Fig. 17 a) and DNA fragmentation (Fig. 17 b). However, in U87 cells, inhibition of NFκB signaling only slightly rescued cells from loss of cell viability and DNA fragmentation (Fig. 17 c and d) but the data fell short on statistical significance. The effects of BV6 single treatment were slightly diminished in both cell lines in higher concentrations whereas IFNα single treatment was unaffected. These results suggest that at least in A172 cells, unimpaired NFκB signaling is critical for BV6 and IFNα to synergistically induce cell death. Since inhibition of NFκB signaling was not sufficient to completely block BV6/IFNα-induced cell death, we assumed additional molecular mechanisms.

3.3.4 BV6/IFNα-cotreatment induces expression of NFκB-responsive genes

To further examine the effect of BV6/IFN-cotreatment on NFκB signaling and to identify whether BV6 and IFNα cooperate to induce NFκB activation we used A172 cells stabily expressing an inducible NFκB-responsive GFP reporter and detected GFP fluorescence after BV6 and IFNα single treatment and BV6/IFNα-cotreatment using flow cytometry. The stable A172 NFκB reporter cells were kindly provided by I. Eckhardt.

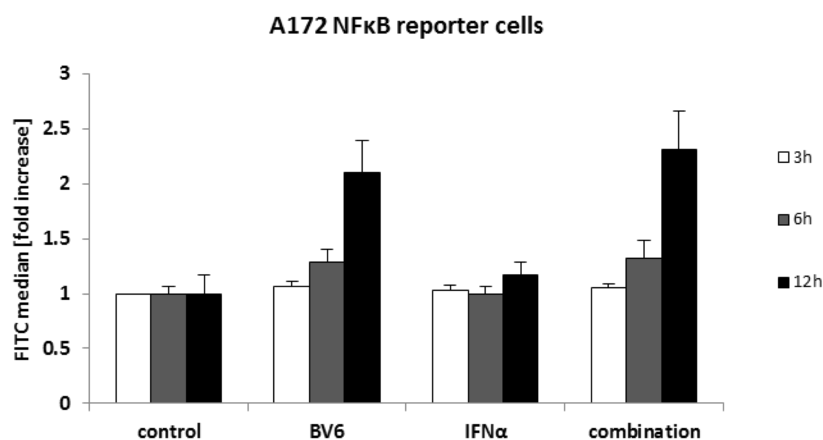

Figure 18 NFκB activity is increased after treatment with BV6 and the combination of BV6 and IFNα

A172 NFκB reporter cells were treated for the indicated time points with BV6, IFNα and the combination of both (2 µM BV6, 5 ng/ml IFNα). Median FITC intensity was measured by flow cytometry and expressed as fold increase. Mean ± S.D. of at least three independent experiments performed in duplicates are shown.

Application of IFNα single treatment had no notable effect on the activation of NFκB as indicated by GFP fluorescence (Fig. 18). In contrast, BV6 single treatment substantially induced NFκB activation after 6 hours and 12 hours. This finding is consistent with the assumed Smac mimetic-dependent accumulation of NIK and subsequent activation of NFκB TFs. Interestingly, BV6/IFNα-cotreatment did not further increase NFκB activation compared to BV6 single treatment after 12 hours.

Taken together, BV6 and IFNα cooperate to deplete cIAP1 and cIAP2 and to induce accumulation of NIK, phosphorylation of IκBα and proteasomal processing of p100. However, inhibition of NFκB signaling was not sufficient to fully rescue cells from dying

but significantly decreased cell death. Moreover, BV6/IFNα-cotreatment did not additionally increase NFκB activation compared to BV6 single treatment. In conclusion, this set of experiments indicates that on the one hand, NFκB activation is required for BV6/IFNα-induced cell death. On the other hand, NFκB activation is not sufficient to fully explain the underlying molecular mechanism and additional pathways may contribute to the treatment.

3.3.5 BV6/IFNα-cotreatment induces cell death independent of extracellular TNFα or TRAIL.

Since autocrine TNFα or other death ligands have been frequently described to be required for SMAC mimetic-induced cell death, we next addressed whether death ligands are involved in the execution of BV6/IFNα-mediated apoptosis.[159,160] First, we used a pharmacological approach to block extracellular TNFα from TNFR1 binding by using the TNFα-blocking antibody Etenercept (Enbrel®). Similarly, we pharmacologically neutralized the interaction of TRAIL, another key inducer of extrinsic apoptosis, with its cognate cellular receptor.[160] With this set of experiments we aimed to address the question whether BV6/IFNα-cotreatment requires extracellular TNFα or TRAIL for the induction of cell death in GBM cells.

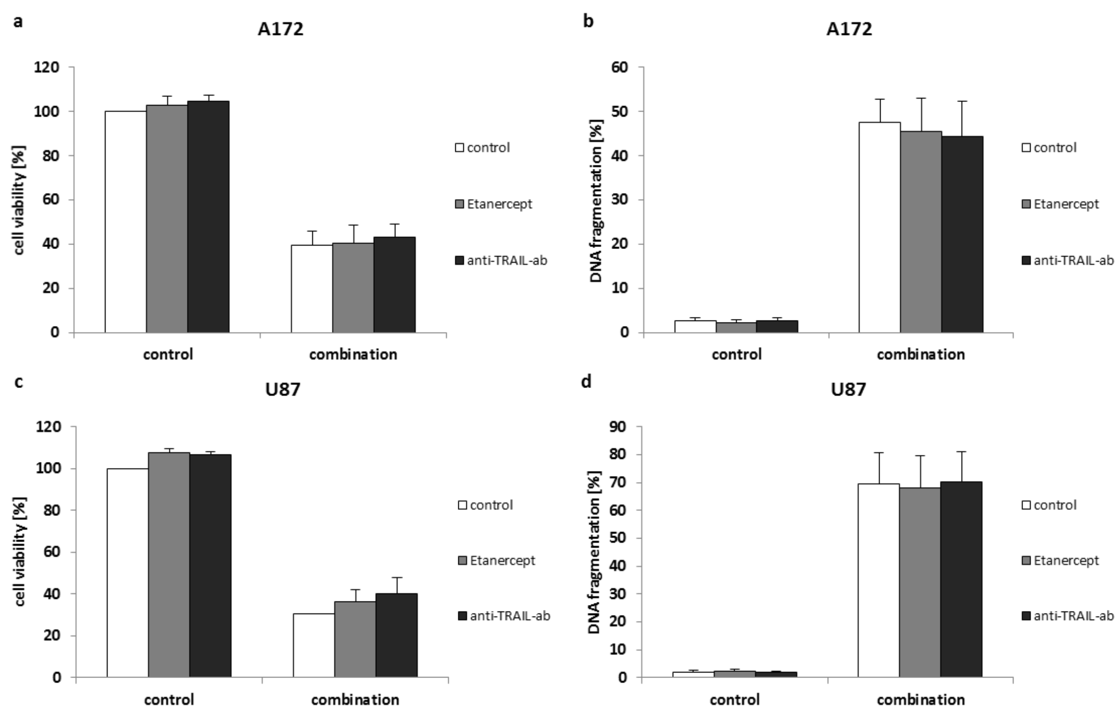

Figure 19 BV6 and IFNα corporately trigger loss of cell viability and DNA fragmentation in glioblastoma cells mainly independent of TNFα and TRAIL

Cells were treated for 72 hours with BV6 and IFNα (2 µM BV6 + 5 ng/ml IFNα for A172, 6 µM BV6 + 10 ng/ml IFNα for U87, referred to as combination) with 10 µg/ml TNFα-blocking antibody Etanercept (Enbrel®) or 1 µg/ml of anti-TRAIL-ab. Cell viability was measured by MTT staining and DNA fragmentation

was assessed by flow cytometry after staining with Nicoletti buffer. Mean ± S.D. of at least three independent experiments performed in triplicates (MTT) or duplicates (DNA fragmentation) are shown. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

Surprisingly, blocking of TNFα and TRAIL did not affect levels of cell death or DNA fragmentation when compared to BV6/IFNα-cotreated cells in the absence of pharmacological inhibitors. These results confirm that in A172 and U87 cells, BV6 and IFNα induce cell death and DNA fragmentation independent of extracellular TNFα and TRAIL.

3.4 Concluding remarks

In summary, this study provides evidence that the Smac mimetic BV6 and IFNα cooperate to induce apoptosis in GBM cells in a synergistic manner as assessed by different cell death assays. Furthermore, the study shows that the cell induction of BV6/IFNα-cotreatment induces activation of canonical and non-canonical NFκB signaling pathways and requires unimpaired NFκB signaling transduction. Moreover, the cotreatment induces cell death independent of soluble TRAIL and TNFα since pharmacological inhibition of either cytokines fails to rescue cells from cell death.

4 DISCUSSION

4.1 BV6 and IFNα cooperate to induce cell death in GBM cells in a synergistic manner

Despite a growing understanding of the complex biology of tumors and intensive multimodal therapy regimes, the prognosis of children with GBM is still poor. Resistance against widely used chemotherapy and radiation is frequently observed and most likely conferred by aberrant cell death regulation including overexpression of IAPs, namely cIAP1, cIAP2 and XIAP.[280,281] Therefore, current attempts to improve the survival of GBM patients aim to target tumor cell resistance and sensitize tumor cells to induction of apoptosis while limiting cellular toxicity.[280] In the last years, research has provided ample support for the assumption that targeting IAPs by small molecular Smac-derived inhibitors, named Smac mimetics, represents a promising strategy for the therapy of GBM and several other human malignancies.[156,166,173,280,282] In line with the notion that Smac mimetics can sensitize malignant cells to apoptotic stimuli, they were extensively studied in combination protocols together with chemotherapeutics, death receptor ligands and cytokines to improve tumor response to existing therapy regimes.[173]

In this study we show for the first time that the small molecular Smac mimetic BV6 synergizes with the human cytokine IFNα to induce cell death in GBM cells in a dose- and time-dependent manner as confirmed by CI calculation (Fig. 11, Table 1). Since IFNs are investigated as an experimental anti-tumor drug in several cancer models, including a broad spectrum of haematological and solid tumors, these studies are of particular interest for future clinical trials.[283,284] In GBM cells, BV6 has been shown to effectively induce cell death and to synergize with the first-line chemotherapeutic Temozolomide, the human monoclonal DR5-antibody Drozitumab and γ-irradiation to trigger apoptosis.[156,285,286] The data obtained in flow cytometry and western blot analysis supports the assertion that BV6/IFNα-cotreated cells mainly die by apoptotic cell death, since the combination increases DNA fragmentation (Fig. 12) and PI positivity (Fig. 13)

in a dose- as well as time-dependent manner. Moreover, western blot analysis confirms procaspase-3 and procaspase-8 cleavage (Fig. 14 e) and application of the broad spectrum caspase-inhibitor zVAD.fmk significantly rescues A172 cells from cell death induction (Fig. 14 a - d). However, additional experiments are necessary to address the question, whether alternative modes of programmed cell death, including necroptosis, may be involved in the BV6/IFNα-induced cell death since zVAD.fmk failed to fully rescue A172 and U87 cells from dying. Moreover, DNA fragmentation and membrane permeabilization, as indicated by PI positivity, but not caspase activation are shared features of late apoptosis and late necroptosis.[287] Hence the disparate decrease in cell death and DNA fragmentation observed in cells treated with zVAD.fmk could be attributed to additional modes of cell death, namely necroptosis. Future studies should aim to incorporate methods to further distinguish between apoptosis and other forms of cell death, for example pharmacological or genetic RIPK1 or RIPK3 inhibition as well as western blot analysis of RIPK1 and RIPK3 expression.

4.2 The induction of cell death by BV6/IFNα-cotreatment depends on NFκB signaling and caspase activation.

Mechanistically, we show that the synergism of BV6 and IFNα critically depends on NFκB signaling and caspase activation whereas it is independent of paracrine/autocrine TNFα and TRAIL. These conclusions are supported by the following independent lines of evidence:

First, genetic inhibition of NFκB activation by stable expression of a dominant negative IκBα-superrepressor significantly decreased BV6/IFNα-cotreatment-induced cell death and DNA fragmentation in A172 cells (Fig. 16) compared to control cells. Moreover, western blot analysis confirmed engagement of the NFκB pathway by BV6/IFNα-cotreatment-dependent stabilization and accumulation of the upstream kinase NIK and subsequent processing of NFκB p100 to p52 (Fig. 15). Notably, Eckhardt et al. showed that BV6 mediated accumulation of NIK, a well described mediator of non-canonical

NFκB signaling, facilitates activation of both canonical and non-canonical NFκB pathways.[157] This co-activation of both NFκB signaling branches may be mediated via NIK dependent crosstalk.[288] Furthermore, they described that dominant negative IκBα-SR suppresses both canonical and non-canonical NFκB signaling. Interestingly, BV6/IFNα-cotreatment did not significantly increase NFκB activation compared to BV6-single treated cells as indicated by GFP fluorescence (Fig. 18). Thus, BV6-treatment may render GBM cells susceptible to additional apoptotic stimuli, such as IFNα, in a NFκB-dependent manner. Recently, Smac mimetic-induced NFκB activation has been reported to sensitize GBM cells to pro-apoptotic stimuli, namely Temozolomide and γ-irradiation.[156,277] This is noteworthy, regarding the widely held view that NFκB is well known for its anti-apoptotic function.[179,230,289] However, it is interesting to note that non-canonical NFκB activation has been reported to induce anti-apoptotic mechanisms, including cell elongation, migration and invasion in GBM cells treated with non-toxic BV6-concentrations.[277] There is some evidence that Smac mimetics may engage apoptotic as well as non-apoptotic pathways in GBM cells in a concentration-dependent manner.[277] Further research is necessary to address the question whether Smac mimetic-induced activation of the canonical and non-canonical NFκB pathway engages apoptotic and non-apoptotic effects in GBM cells in different manners and how activation of NFκB renders GBM cells susceptible to death signals.[216]

4.3 Induction of cell death by BV6/IFNα is independent of TNFα and soluble TRAIL

Second, we showed that BV6/IFNα-induced cell death is independent of extracellular TNFα and TRAIL since pharmacological inhibition fails to protect GBM cells from cell death induction (Fig. 19). This finding is of particular interest since BV6 single treatment has been reported to critically depend on paracrine/autocrine TNFα/TNFR1-signaling in several human malignancies.[279,283,290,291] Of note, in 2011, Tenev et al. reported the discovery of a signaling platform, termed the Ripoptosome, inducing apoptosis in

cIAP1/2-depleted cells independent of TNFα, TRAIL or CD95.[292] The complex consists of RIPK1, FADD and caspase-8 and facilitates caspase-8 activation as well as caspase-independent necroptosis in cells irrespective of their ability to produce TNFα. Thus, Smac mimetic-induced Ripoptosome formation and subsequent cell death in BV6/IFNα-cotreated cells may play a role in GBM cells, since cell death occurs largely independent of TNFα/TNFR1-signaling and TRAIL ligation. Additionally, studies with chronic lymphoid leukemia cells supports the assumption that defective Ripoptosome formation renders cells resistant to Smac mimetic-induced cell death.[293] However, since we showed that BV6/IFNα-cotreatment critically depends on NFκB activation, expression of additional target genes may be involved. Just recently, Eckhardt et al. identified the TRAIL-receptor DR5 as a critical mediator for BV6-induced apoptosis in GBM cells that die independently of paracrine/autocrine TNFα/TNFR1-signaling.[157] Furthermore, the study indicates that DR5 mediates apoptosis independent of soluble TRAIL but partially TRAIL-dependent, since genetic silencing of TRAIL partially rescues cells from BV6-mediated cell death. Notably, Roesler et al. demonstrated recently that genetic silencing of TRAIL by small interfering ribonucleic acid (siRNA) knockdown significantly reduced BV6/IFNα-induced cell death in A172 cells.[294] Furthermore, genetic silencing of DR5 similarly protected A172 cells against BV6/IFNα-imposed cell death.[294] Since TRAIL is expressed both as a soluble, secreted protein as well as a membrane-bound protein, pharmacological inhibition of soluble TRAIL may be insufficient to inhibit TRAIL-dependent induction of cell death whereas genetic silencing of the soluble and the membrane-bound form significantly rescues A172 cells from cell death. Similar disparate effects of soluble and membrane-bound TRAIL were reported in other tumor entities strengthening the assumption that both forms of TRAIL induce different signaling pathways and that pharmacological inhibition is not sufficient to study the TRAIL-dependent induction of cell death *in vitro*.[295] Moreover, IFNα has been reported to induce the expression of TRAIL in a broad spectrum of cell lines.[296–299] With that in mind, further research should address the question, whether TRAIL-induced RIPK1/FADD/caspase-8 assembly is required for BV6/IFNα-induced cell death and whether enhanced expression of DR5 is critically involved in BV6/IFNα-mediated cell death.

4.4 Additional NFκB target genes might be involved in BV6/IFNα-induced cell death

Further research should aim to identify additional NFκB target genes that might play a role in BV6/IFNα-induced cell death such as IRF1, which was found to promote apoptosis in several cell lines and to transcriptionally induce genes encoding caspase-1, caspase-7, caspase-8, FasL and TRAIL.[261,267,270,272,300] IRF1 is known as a NFκB target gene and has been reported to be upregulated by various NFκB stimuli.[301] Furthermore, it was shown to cooperate in concert with NFκB to regulate the expression of NFκB target genes.[302] Eckhardt et al. identified IRF1 as a key dual regulator for BV6-mediated cell death and secretion of proinflammatory cytokines in cancer cells of different entities.[303] Additionally, the authors revealed that IRF1 serves as a transcriptional activator that is essential to fully engage the BV6-triggered apoptotic program and that IRF1 silencing significantly rescues tumor cells from cell death.[303] Moreover, Bake et al. identified IRF1 as a pivotal transcription factor for BV6/IFNα-mediated cell death in acute myeloid leukemia.[283] However, IRF1 is known to play critical functions in a broad spectrum of cellular signaling pathways, including immune responses, TLR-signaling, viral infection, immune cell development and oncogenesis.[261] In cancerous cells, IRF1 was shown to enhance expression of major inflammatory cytokines, including TNFα, IL-6 and IL-8 following BV6 treatment.[303] Those cytokines were shown to induce cancer-related inflammation and immune escape thus promoting cancer growth and disease progression.[304–306] Given the versatile functions performed by IRF1 in a large number of signaling pathways, further studies are necessary to address the question, whether IRF1 is implicated in BV6/IFNα-induced cell death and tumor immunity.

4.5 Summary and further outlook

In summary, our study demonstrates that BV6/IFNα-cotreatment induces cell death in GBM cells in a highly synergistic manner. The cell death is characterized by a cellular loss of viability, induced DNA fragmentation and PI positivity. Mechanistically, we demonstrated that the induction of cell death requires NFκB signaling as well as the activation of caspases but is independent of extracellular TNFα and soluble TRAIL. Our study contributes to a better understanding of Smac mimetics and Smac mimetic-based combination protocols in GBM cells. Given the natural limitations of *in vitro* models, further studies could aim to verify the findings of this study *in vivo*. Since IAP antagonists are currently under evaluation in clinical trials, these findings may have broad implications for the successful application of Smac mimetics as clinical cancer therapeutics.[173]

4 Discussion

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SCHRIFTLICHE ERKLÄRUNG

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin der Johann Wolfgang Goethe-Universität Frankfurt am Main zur Promotionsprüfung eingereichte Dissertation mit dem Titel

Evaluation of the bivalent Smac mimetic BV6 and Interferon α as a combination treatment in Glioblastoma multiforme

In im Zentrum der Kinder- und Jugendmedizin am Institut für Experimentelle Tumorforschung in der Pädiatrie unter der Betreuung und Anleitung Prof. Dr. med. Simone Fulda mit Unterstützung durch Dr. Ines Eckhardt ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe. Darüber hinaus versichere ich, nicht die Hilfe einer kommerziellen Promotionsvermittlung in Anspruch genommen zu haben.

Ich habe bisher an keiner in- oder ausländischen Universität ein Gesuch um Zulassung zur Promotion eingereicht*. Die vorliegende Arbeit wurde bisher nicht als Dissertation eingereicht.

Vorliegende Ergebnisse der Arbeit wurden in folgendem Publikationsorgan veröffentlicht:

Roesler S, Eckhardt I, Wolf S, Fulda S. Cooperative TRAIL production mediates IFNα/Smac mimetic-induced cell death in TNFα-resistant solid cancer cells. Oncotarget. 2016;7(4):3709-25.

_______________________ _____________________________

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2016	Roesler S, Eckhardt I, Wolf S, Fulda S. Cooperative TRAIL production mediates IFNα/Smac mimetic-induced cell death in TNFα-resistant solid cancer cells. Oncotarget. 2016;7(4):3709-25.
	Bruch HR, Dencausse Y, Heßling J, et al. CONIFER - Non-Interventional Study to Evaluate Therapy Monitoring During Deferasirox Treatment of Iron Toxicity in Myelodysplastic Syndrome Patients with Transfusional Iron Overload. Oncol Res Treat. 2016;39(7-8):424-31.

SOZIALES ENGAGEMENT

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	Vorstandsmitglied und langjährig aktives Mitglied im Musikverein Harmonie Neuses
	Musiklehrer für die musikalische Jugendförderung
10/2008	3-wöchiger Aufenthalt in **Moschi, Tansania** mit dem Verein „Helfen macht Schule“

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