The NF-κB signaling pathway in melanoma cells and implications for its therapeutic modulation

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2 Abbreviation

±SEM	standard error of the mean
A375	human melanoma cell line generated from cutaneous
ABC-	ATP-binding cassette transporter
Aberration	full word
AML	acute myeloid leucemia
Apaf-1	apoptosis activating factor 1
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
BAFF	B cell activating factor
CBP	CREB binding protein
CCL2	monocyte chemotactic protein 1 /Chemokine (C-C motif)
CDK	cyclin dependent kinase
cDNA	complementary DNA
CXCL1	chemokine (C-X-C motif) ligand 1
CXCL8	interleukin 8 / chemokine (C-X-C motif) ligand 8
DD	death domain
DICT	darcabacin
DISC	death inducing complex
DMSO	Dimethyl sulfoxide
DNA	desoxyribonuclein acid
DNA	desoxyribonuclein acid
DNA-DSB	DNA double strand breaks
DR	death receptor
DR	death receptor
DTT	dithiothreitol
DUSP6	dual specificity phosphatase 6
ECM	extra cellular matrix
EDTA	ethylenediaminetetraacetic acid
EMA	European Medicine Agency
FDA	US Food and Drug Administration
FSC	fetal calf serum
g	gravtation
H1299	human non-small cell lung carcinoma cell line
HeLa	human cancer cell line

HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cells
IAP	inhibitor of apoptosis protein
ICAM-1	intercellular adhesion molecule 1
IKK	inhibitor of κB kinase
IL-1	interleukin 1
IL-6	interleukin 6
IRF-3	interferon regulatory factor 3
IκB	inhibitor of κB
LMP1	latent membrane protein-1
LOX	human melanoma cell line generated from lymph node
LPS	lipopolysaccharide
MC1R	melanocortin-1 receptor
MMNH	human melanoma cell line generated from cutaneous
MMP	matrix metalloproteinase
MMR	miss match repair
MRP	multidrug-resistance-associated protein
MV3	human melanoma cell line generated from cutaneous
NEMO	NF-κB essential modulator/ IKKγ
NF-κB	nuclear factor-'kappa-light-chain-enhancer' of activated B
NGF	nerve growth factor
NIK	NF-κB inducing kinase
NLS	nuclear localisation sequence
PARP1	poly(ADP-ribose)-polymerase 1
PBS	phosphate buffered saline
PDK1	phosphoinositide-dependent kinase 1
PI3K	phosphatidylinositol-3-kinase
PIASγ	protein inhibitor of activated STAT γ
PIP_2	phosphatidylinositol-(3,4)-P_2
PIP_3	phosphatidylinositol-(3,4,5)-P_3
PUMA	p53 upregulated modulator of apoptosis
RGP	radial growth phase
RHD	Rel-homology domain
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
RT-PCR	revers transcription polymerase chain reaction
RT-PCR	reverse transcription - polymerase chain reaction
RXRα	retinoid x receptor alpha

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	small interfering RNA
SUMO	small ubiquitin-like modifier
TAK1	transforming growth factor β-activated kinase 1
TCR	T cell receptor
TLR	Toll-like receptor
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRAF	TNF receptor associated factor
TRAIL	TNF related apoptosis inducing ligand
U-2 OS	human osteosarcoma cell line
UVR	ultra violet radiation
UVR	ultra violet radiation
VCAM	vascular adhesion molecule 1
VEGF	vascular endothelial growth factor
VGP	vertical growth phase
XIAP	X-linked inhibitor of apoptosis

3 Abstract

One of the major challenges in cancer therapy is to overcome drug resistance. Melanoma cells are an illustrative example for this notion, as metastasized melanoma is almost universally resistant against chemotherapy. The NF-κB signaling pathway is constitutively active and plays a crucial role for drug resistance in melanoma cells.

This work starts from the observation that doxorubicin leads to profound activation of NF-κB in two different melanoma cell lines, while several other chemotherapeutics with different modes of action did not activate this pathway. Likewise, NF-κB dependent transcription of mediators, which are thought to be involved in tumor progression, was increased by doxorubicin. Notably, the strongest NF-κB activation was detected at a concentration of 1 µM doxorubicin corresponding to the concentrations reached when malignant tumors are treated *in vivo*. In search for improved therapeutic strategies, doxorubicin-induced NF-κB activity was analyzed following inhibition of either of the two major kinases of the NF-κB pathway, IKKα and IKKβ, by small molecules compounds. Inhibition by the novel IKKα inhibitor (BAY 32-5915; 8 hydroxyquinoline-2-carboxylic acid), which was identified from a large library, did not affect doxorubicin-induced NF-κB activation. However, doxorubicin-induced NF-κB activation was completely abrogated when the IKKβ-inhibitor KINK-1 was used. Moreover, inhibition of IKKβ, but not IKKα, in combination with doxorubicin significantly increased the apoptotic response of melanoma cells. In contrast, cisplatin, which did not activate NF-κB, failed to enhance apoptosis when used concomitantly with either of the IKK inhibitors. Complementary, while silencing of IKKβ using siRNA notably reduced doxorubicin-induced NF-κB activity, silencing of IKKα had only moderate effects on this pathway and, consecutively, downstream gene transcription. Furthermore, depletion of IKKα did not influence the synthesis of several apoptosis-related proteins or cellular functions such as migration, proliferation or apoptosis. Accordingly, IKKα reduction did not increase the susceptibility of melanoma cells to doxorubicin. In addition, blocking of ATM by small molecule

inhibitors also increased doxorubicin-induced apoptosis through NF-κB inhibition in melanoma cells.

In summary, IKKα reduction is not sufficient to improve the response of melanoma cells to chemotherapy with doxorubicin, while IKKβ inhibition results in profound increase of susceptibility. ATM may be an interesting player in the complex molecular interactions governing doxorubicin-induced apoptosis in melanoma cells.

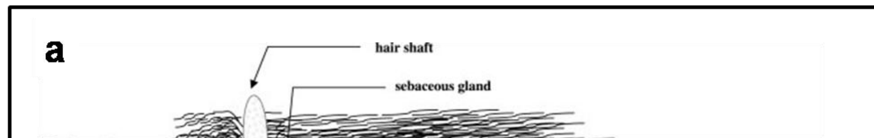
4 Introduction

4.1 Melanoma

Melanoma, the deadliest type of skin cancer, arises – with few exceptions - from pigment cells (melanocytes) of the skin. The human skin is composed of three primary layers: the epidermis (the epithelium of the skin), the dermis (the fibrous connective tissue) and the subcutaneous fatty tissue. Melanocytes originate from the neural crest and localize to the basal layer of the epidermis and a number of extracutaneous tissues during embryogenesis (Markovic et al. 2007; Sommer 2011). The most important function of melanocytes is the production of melanin. The photoprotective pigment melanin is synthesized within the membrane-bound melanosomes and then transferred to the epidermal keratinocytes, where the melanin granules become protectively arranged over the nucleus to shield the cell´s sensitive DNA from ultraviolet (UV) radiation (Markovic et al. 2007). Melanin is a broadband UV radiation absorbent that neutralizes UV-generated free radicals (Bustamante et al. 1993; Brenner and Hearing 2008).

Notwithstanding, clear evidence for a decisive role of UV-induced DNA-damage in the pathogenesis of non-melanoma skin cancer (i.e., basal cell carcinomas and squamous cell carcinomas, the so-called “white” skin cancer), the risk of melanoma development is not quite as clearly correlated with UV-exposure. It is thought that a complex interplay of UV-radiation and genetic factors underly its pathogenesis, because melanoma may arise in both sun-exposed and non-exposed skin (Maddodi and Setaluri 2008). Some melanomas (approximately 25% of all melanomas) develop from pre-existing pigmented lesions called melanocytic nevi or moles (Clark 1991). Melanomas restricted to the epidermis are called in situ melanomas. Such lesions are in the radial growth phase (RGP) and have not (yet) penetrated the epidermal basement membrane (Clark 1991). At this stage, surgical excision usually cures the disease. However, once melanomas enter the vertical growth phase (VGP), which may follow penetration of the basement membrane, they grow invasive and achieve the capacity to metastasize. Once melanomas have formed

metastases in distant organs, the 5 year survival of the patients decreases to < 10 % (Balch et al. 2001; Bene et al. 2008). Metastasized melanomas are usually highly resistant against radiation and chemotherapy (Soengas and Lowe 2003).



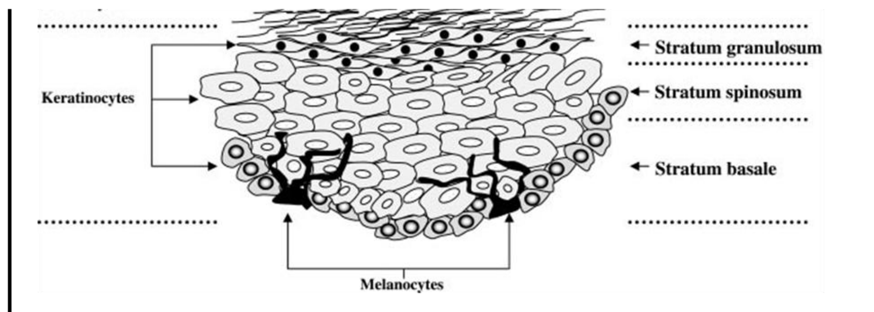


Figure 1: Anatomy of the skin (from Costin and Hearing 2007).

(a) Different layers and components of the skin without the components of the extracellular matrix (ECM). (b) The layers of the epidermis. Melanocytes are located within the basal layer of the epidermis.

According to recent data published by the Robert Koch institute, the incidence of melanomas in Germany has increased three fold since the 1980's, while the mortality remained rather constant. This apparent discrepancy has been attributed primarily to intensive information of the public and early diagnosis (Kaatsch et al. 2012). In the year 2008, nearly 18,000 new cases

have been registered in Germany alone, most of them in an early stage (Kaatsch et al. 2012).

Dacarbacin (DTIC), an alkylating drug which generates O^6-methylguanine adducts, was the only drug approved for systemic treatment of metastatic melanomas for more than three decades – despite tremendous efforts in preclinical and clinical research (Serrone et al. 2000). Given that DTIC achieved response rates of only approximately 10 % and the overall survival of the patients could not be improved significantly, the success of such treatment was poor. Very recently, this sobering situation has changed somewhat, as two new compounds were approved for the treatment of metastatic melanoma in 2011: the selective B-RAF inhibitor, Vemurafenib, and the T-cell activator, Ipilimumab. For the first time, these drugs achieved an improvement overall survival of the patients by several months (Eggermont and Robert 2011). Unfortunately, melanoma cells may become resistant against Vemurafenib through activation of receptor tyrosine kinase (RTK) dependent survival pathways or reactivation of the RAS/RAF/MEK/ERK pathway following N-RAS upregulation (Nazarian et al. 2010). Thus, further research is necessary to better understand the mechanisms of melanoma chemoresistance.

4.2 Apoptosis

A characteristic feature of cancer including melanoma is a shift of the equilibrium between proliferation and apoptosis towards proliferation (Hanahan and Weinberg 2000). Thus, a central aim of chemotherapy is the induction of apoptosis in cancer cells. Apoptosis is a tightly regulated cell death program that depends on activation of caspase cascades (Hengartner 2000). Caspases, a family of cysteine proteases, are ubiquitously expressed as inactive precursors and can be activated by the extrinsic (death receptor-dependent) or intrinsic (mitochondrial) pathway of apoptosis.

4.2.1 Extrinsic apoptosis

The extrinsic pathway of apoptosis is initiated through membrane-bound death receptors (DR). The transmembrane DR belong to the tumor necrosis factor receptor (TNFR) superfamily; each TNFR can be triggered by specific

tumor necrosis factor (TNF)-related ligands. The Fas receptor (CD95R) and the TNFR1 pathway with their ligands FasL (CD95) and TNFα, respectively, have been studied most extensively. However, another 18 ligands and 28 receptors of the TNF/TNFR superfamily have been discovered (Hengartner 2000). The exact contribution to apoptotic responses of some of these receptor/ligand pairs is still a matter of ongoing research. Once DR-ligands bind, their receptors come into close proximity with and form an intracellular binding site for death domain (DD)-containing adapter proteins. Each receptor has specific adapter proteins, although there appears to be some redundancy. The complex of ligand, receptor and adapter protein, called the death-inducing signal complex (DISC), recruits caspase-8. The subsequent autoproteolytic cleavage of caspase-8 activates effector caspases, which then execute cellular apoptosis (Hengartner 2000; O'Brien and Kirby 2008; Wong 2011).

4.2.2 Intrinsic apoptosis

The intrinsic apoptosis pathway is initiated within the cells by internal stimuli originating from DNA-damage, hypoxia, oxidative stress or chemotherapeutic treatment. The intrinsic apoptosis pathway results in the release of mitochondrial cytochrome c, a process that is regulated by proteins of the Bcl2-family. The Bcl2-family comprises pro-apoptotic (Bax, PUMA, NOXA) and anti-apoptotic (Bcl2, Bcl_{XL}) members. These proteins are located at mitochondrial membranes, where they regulate membrane permeability through manipulating the membrane potential. For example, the anti-apoptotic Bcl2-family members Bcl2 and Bcl_{XL} prevent opening of transient mitochondrial pores for cytochrome c release, whereas the pro-apoptotic members of the Bcl2-family block the function of the anti-apoptotic members. Thus the stoichiometric relation of the Bcl2-family members is crucial for apoptosis induction (O'Brien and Kirby 2008). Once cytochrome c is released into the cytosol, it associates with apoptosis activating factor-1 (Apaf-1) and caspase-9, thus forming a complex called the apoptosome which then activates downstream caspases (O'Brien and Kirby 2008; Wong 2011).

The extrinsic pathway results in activation of caspase-8 and the intrinsic pathway in activation of caspase-9, both pathways converge at the activation of

the terminal effector caspase-3. Caspase-3 releases deoxyribonuclease by cleavage of its inhibitor, thus executing nuclear apoptosis. Likewise, other downstream caspases become activated resulting in cleavage of protein kinases, cytoskeletal proteins, DNA repair proteins and inhibitory subunits of endonucleases. All these cleavage events result in the typical morphological appearance of apoptotic cells, such as DNA fragmentation and membrane blebbing (Ghobrial et al. 2005).

Both apoptosis pathway are not completely separated from each other and there are several inter-dependent regulatory mechanisms connecting the two. To mention just one example for this cross-talk: caspase-8 can cleave Bid, a pro-apoptotic member of the Bcl2-family, which then promotes cytochrome c release (Grossman and Altieri 2001).

4.3 Chemoresistance of melanoma cells

Induction of apoptosis in cancer cells is arguably the most important aim of chemotherapeutic treatment. However, melanoma cells are highly resistant to chemotherapy. This unfortunate trait is based on several molecular mechanisms, some of which are mutually enhancing (Nazarian et al. 2010):

4.3.1 Drug efflux

Two classes of ATP-dependent drug transporter proteins are involved in tumor cell resistance, the P-glycoprotein family and the multidrug-resistance-associated proteins (MRPs). The expression of the ABCB5 protein, a transporter of the P-glycoprotein family, was shown at the surface of melanoma cells, where it counteracted intracellular doxorubicin accumulation (Frank et al. 2003; Frank et al. 2005). In support of this notion, blocking of ABCB5 resulted in intracellular accumulation of doxorubicin and the LD_{50} of doxorubicin was decreased by ~43 % (Frank et al. 2005). In contrast, MRPs are expressed by melanoma cells, albeit their modulation by chemotherapeutic treatment appears to be less obvious (Grossman and Altieri 2001).

4.3.2 Detoxification

Glutathione-S-transferase is involved in detoxification of many drugs through conjugation to glutathione. Among others, this enzyme detoxifies alkylating agents such as DTIC or temozolomide. However, although the inhibition of glutathion-S-transferase increased the susceptibility of A375 melanoma cells to DTIC and its derivative temozolomide *in vitro* (Tentori et al. 2011), a correlation of glutathion-S-transferase levels and the clinical response of melanoma patients could not be demonstrated (Grossman and Altieri 2001).

4.3.3 DNA repair

Most chemotherapeutics modify or disturb the DNA integrity. This damage is sensed by DNA-associated factors which activate DNA repair mechanisms. Indeed, drug resistant melanoma cell lines feature increased DNA repair (Grossman and Altieri 2001). In addition, a loss of mismatch repair (MMR) proteins was detected in 30 % to 70 % of melanomas, although a correlation between intactness of DNA repair mechanisms and clinical course could not be demonstrated (Bradbury and Middleton 2004).

4.3.4 Apoptosis deregulation

Advanced melanomas are highly refractory to apoptosis induction, even compared to other cancer cell types. This trait is owed to intrinsic and acquired apoptosis defects (Soengas and Lowe 2003). Indeed, even normal melanocytes have a considerable resistance power in an environment where apoptosis is induced in neighbor cells (Box and Terzian 2008).

The extrinsic apoptosis pathway can be blocked by downregulation of death receptors or by surface expression of decoy receptors, with the latter binding death ligands without inducing intracellular apoptosis (Grossman and Altieri 2001). Moreover, increased synthesis of a structural homologue of caspase-8 (FLIP) that lacks proteolytic activities also inhibits the extrinsic apoptosis pathway (Grossman and Altieri 2001).

An important tumor suppressor is p53. It becomes activated by DNA-damage and induces cell cycle arrest by promoting p21 synthesis which blocks

the cyclin/CDK complex. The latter is important for the G1/S-phase transition (Gartel and Tyner 2002). Furthermore, p53 can also induce apoptosis by suppressing synthesis of anti-apoptotic proteins, like Bcl2, or inducing synthesis of pro-apoptotic proteins, such as Bax (O'Brien and Kirby 2008). Additionally, p53 can modulate the extrinsic apoptosis pathway through upregulation of the Fas receptor (O'Brien and Kirby 2008). A hallmark of many cancer cells is the mutation or loss of heterozygosity of p53, but this occurs only in 0 % to 10 % of melanomas (Box and Terzian 2008). Interestingly, melanocytes, the precursors of melanoma cells, feature an intrinsic attenuation of p53 dependent apoptosis, because after DNA-damage they have to survive for melanin production (Box and Terzian 2008). In addition, keratinocytes secrete several mediators, which stimulate the synthesis of anti-apoptotic proteins in melanocytes (Soengas and Lowe 2003).

Inhibitors of apoptosis proteins (IAP) represent another family of critical regulators of induced apoptosis. These proteins are able to halt apoptosis downstream of many apoptosis-inducing stimuli: TNFα, FasL, UV-radiation, viral infection, cytochrome c, growth factor retraction, caspases or chemotherapeutic drugs (LaCasse et al. 1998). For example, survivin (an IAP family member) is not expressed in melanocytes but is found in melanoma cells. In the latter, survivin blocks apoptosis at the level of caspase-9 and its inhibition results in apoptosis and cell cycle defects (Grossman and Altieri 2001).

To conclude the brief exemplary selection of factors influencing apoptosis in melanoma cells, DNA-methylation of genes whose products are involved in apoptosis results in reduced Apaf-1 levels. This downregulation impairs formation of the apoptosome (Soengas and Lowe 2003).

4.4 Signaling pathways in melanoma

While the dysregulations of signaling pathways in melanoma cells are very complex, past and current research has emphasized three central pathways involved in melanoma progression and chemoresistance: the PI3K-AKT, the RAS/RAF/MEK/ERK and the NF-κB pathway. All three are constitutively active in many melanoma cells.

4.4.1 The PI3K-AKT pathway

The phosphatidylinositol-3 kinase (PI3K)-AKT pathway is regulated by the activity of the PI3K which is activated by receptor tyrosine kinases (RTK), G-protein coupled receptors and GTP binding of RAS proteins (Davies 2012). The PI3K phosphorylates membrane lipids to generate phosphatidylinositol-(3,4) -P_2 (PIP_2) and phosphatidylinositol-(3,4,5)-P_3 (PIP_3). PIP_2 and PIP_3 translocate pleckstrin homology (PH) domain containing proteins to the inner aspect of the plasma membrane. This recruitment includes AKT, which is phosphorylated by another PH domain containing protein, the phophoinositide-dependent kinase 1 (PDK1) (Madhunapantula and Robertson 2009).

The AKT isoform AKT3 is important for melanoma progression, and 60 % to 70 % of sporadic melanomas have an increased activity of it (Madhunapantula and Robertson 2009). AKT3 regulates multiple processes governing apoptosis and proliferation. For example, AKT3 inactivates Bad, a pro-apoptotic member of the Bcl2-family, it enhances the NF-κB transcription activity, and it phosphorylates the GTPase Rac1. Additionally, AKT can also phosphorylate B-Raf^{V600E} (Cheung et al. 2008).

An important antagonist of AKT is the tumor suppressor PTEN, a phosphatase that also acts as lipid phosphatase and hydrolyzes PIP3 and thus inhibits the activation of AKT (Madhunapantula and Robertson 2009). The loss of *PTEN* results in a higher generation of PIP_3 and it increases AKT activation, which protects melanoma cells from apoptosis. Recently, it was shown that only minimal cell death could be induced in *PTEN*-deficient melanoma cells lines, and the progression-free survival of patients with *PTEN* deletions was shorter compared to patients with melanoma containing normal *PTEN* (Davies 2012).

In addition, mice with conditional melanocyte-specific B-Raf^{V600E} expression developed non-invasive melanocytic hyperplasias but no invasive melanoma. Only when *Pten* was silenced in addition to the *B-Raf* mutation, these mice developed invasive melanomas (Dankort et al. 2009). Resistance of melanoma cells against B-RAF-inhibitors seems to be augmented by activation of the PI3K-AKT pathway. As a consequence, the combined treatment with

inhibitors of RAS/RAF/MEK/ERK and PI3K-AKT might prove beneficial for melanoma treatment (Gopal et al. 2011).

4.4.2 The RAS/RAF/MEK/ERK pathway

The RAS/RAF/MEK/ERK pathway is a conserved kinase cascade in eukaryotes. It is involved in the regulation of cell proliferation, differentiation and survival in response to extracellular signals. These extracellular signals, hormones, cytokines or growth factors interact with their receptors and activate small G-proteins of the RAS family. Activated RAS recruits and activates RAF proteins at the cell membrane (Davies et al. 2002). The RAF family includes three serine/threonine kinases, RAF-1, B-RAF and A-RAF. B-RAF has a high basal activity and needs less phosphorylation events for full activity (Mason et al. 1999). Active B-RAF activates MEK1/2, active MEK1/2 activates ERK1/2, and activated ERK1/2 affects downstream transcription factors to induce processes like differentiation, proliferation, growth or apoptosis. The deregulation of this signal cascade can initiate tumor development (Davies et al. 2002). A gain-of-function mutation in the *B-RAF* gene by a transversion of nucleotide 1799 from thymidine to adenosine is observed in more than 90 % of all *B-RAF* mutations (Davies et al. 2002). This phosphomimetic mutation renders constitutive activation of B-RAF^{V600E} by substitution of amino acid valine (V600) to glutamine (E600) during protein biosynthesis (Garnett and Marais 2004; Wan et al. 2004). *B-RAF* is mutated in 60 % to 70 % of malignant melanoma and is associated with tumors developing after low UV-radiation, younger age of the patients at the time of presentation and melanocortin-1 receptor (MC1R) variants (Davies et al. 2002; Landi et al. 2006).

The selective B-RAF^{V600E} inhibitor, Vemurafenib, is one of the first approved biochemical drugs which caused a prolonged survival for melanoma patients (Eggermont and Robert 2011). Vemurafenib leads to reduction of the risk for death and tumor progression compared with DTIC in stage III or VI melanomas with mutated B-RAF^{V600E} (Chapman et al. 2011). Unfortunately, melanoma cells may develop new mechanisms during Vemurafenib treatment to counteract the impact of the drug (Nazarian et al. 2010). The constitutive activation of B-RAF^{V600E} leads not only to constitutive activation of ERK1/2, but

also triggers constitutive activation of the NF-κB pathway (Liu et al. 2007). Interestingly, in preclinical studies it has been shown that inhibition of NF-κB, but not of ERK signaling, confers anti-tumor activity *in vivo.* Targeting the RAS/RAF/MEK/ERK pathway alone is ineffective for melanoma therapy when the NF-κB pathway is not targeted simultaneously (Yang et al. 2009).

4.4.3 The NF-κB pathway

The nuclear factor-'kappa-light-chain-enhancer' of activated B cells (NF-κB) transcription factors were discovered more than 25 years ago by identification of proteins shuttled between cytoplasm and nucleus. They regulate the transcription of a large number of genes (Gilmore and Temin 1988). The constitutive activation of the NF-κB signaling pathway has been detected in many cancers and is associated with inflammatory diseases (Li and Verma 2002; Gilmore 2006). In cancer cells, NF-κB activity promotes proliferation, anti-apoptosis, angiogenesis, motility and migration (Nakanishi and Toi 2005). Additionally, NF-κB activity can result from chemotherapeutic treatment or irradiation. Such “paradoxical” activation of NF-κB leads to suppression of the apoptotic potential of anti-cancer therapy (Kim et al. 2006). Without activation, inactive NF-κB is bound in the cytoplasm to the inhibitor of κB (IκB). Degradation of IκB by the inhibitor of κB kinase (IKK) complex frees NF-κB enabling it to enter the nucleus and initiate the transcription of NF-κB-dependent genes (Zandi et al. 1998).

4.4.3.1 The NF-κB family

The NF-κB family has five members, REL-A (p65), NF-κB1 (p105/p50), NF-κB2 (p100/p52), c-REL and REL-B, which form homo- and heterodimers. They all share a structurally conserved N-terminal Rel-homology domain (RHD) which contains a nuclear-localization sequence (NLS) and is involved in sequence-specific DNA binding and interaction with the inhibitors of NF-κB (IκB). The REL-A, c-REL and REL-B proteins (REL-subfamily) have a transactivation domain within the sequences C-terminal of the RHD. NF-κB1 and NF-κB2 are synthesized as inactive precursors, p105 and p100, respectively (Siebenlist et al. 1994).

4.4.3.2 The inhibitor of NF-κB (IκB) family

The IκB family consists of IκBα, IκBβ, IκBε and Bcl-3. Bcl-3 is atypical for this IκB family, because this nuclear protein features both transactivation and transrepressor functions (Maldonado and Melendez-Zajgla 2011). The binding of IκB proteins to REL-subfamily proteins masks the NLS and keeps the transcription factors in the cytoplasm. The IκB proteins contain ankyrin-repeat motifs in their C-termini, which mediate the protein-protein interactions. The NF-κB precursors p105 and p100 also contain ankyrin-repeats which act similar to IκB proteins (Perkins 2007). IκB is phosphorylated at specific N-terminal serine residues (IκBα at Ser-32 and Ser-36) by upstream kinases followed by ubiquitination through the E3 ubiquitin ligase, β-TrCP. It can then be degraded by the 26S proteasome (Karin and Ben-Neriah 2000). The processing of the NF-κB precursors, p105 to p50 and p100 to p52, is also regulated by the ubiquitin-proteasome mechanism (Chen 2005). Thus, the NLS of NF-κB factors is unmasked and the freed NF-κB can enter the nucleus and bind to their consensus sequence and initiate the transcription of their target genes.

4.4.3.3 The inhibitor of κB kinase (IKK) complex

The phosphorylation of the IκB proteins and thus their degradation is usually induced by the inhibitor of κB kinase (IKK) complex, which can be activated by a wide range of stimuli (Li et al. 2002). The IKK complex consists of three IKK subunits: the catalytic subunits IKKα, also called CHUK, and IKKβ and the regulatory subunit IKKγ, also called NF-κB essential modulator (NEMO) (Connelly and Marcu 1995; Zandi et al. 1997; Rothwarf et al. 1998). Phosphorylation of IKKα and IKKβ at serine S176/S180 or S177/181, respectively, leads to a conformational change in the activation loop and to their catalytic activity (Mercurio et al. 1997; Regnier et al. 1997). The regulatory IKKγ is absolutely required for the canonical NF-κB pathway, whereas the requirement of IKKβ depends on the acting stimuli. IKKβ can be substituted by IKKα when NF-κB is stimulated by IL-1 (Li et al. 2002; Solt et al. 2007). Three distinct pathways leading to activation of NF-κB can be distinguished, the canonical, the non-canonical and the atypical NF-κB pathway.

4.4.3.4 The canonical NF-κB pathway

The canonical pathway of NF-κB activation, also known as the classical pathway, can be stimulated by the tumor necrosis factor receptor (TNFR) family, Toll-like receptor/Interleukin (IL)-1 receptor (TLR/IL-1R) family or T cell receptor (TCR) (Li and Verma 2002). The di- or trimerisation of receptors after ligand binding initiates the recruitment and activation of adapter proteins, which activate the IKK complex by ubiquitination of IKKγ at lysine K63. This does not lead to IKKγ degradation but to the recruitment of kinases, which phosphorylate IKKβ of the IKK complex (Li et al. 2002; Chen 2005; Perkins 2007). The active IKK complex then phosphorylates IκB at specific serine residues for its degradation by the S26 proteasome. As a consequence, the NF-κB subunits are freed and induce transcription of their target genes, which include chemokines, cytokines and adhesion molecules and are involved in inflammatory responses and cell survival (Karin and Ben-Neriah 2000; Nishikori 2005). Another mechanism to activate the canonical NF-κB pathway is regulated by the activation of the nuclear kinase ataxia telangiectasia mutated (ATM) by genotoxic stimuli, such as irradiation or chemotherapeutic treatment. In an assembly with poly(ADP-ribose)-polymerase 1 (PARP1) and the SUMO-1 ligase PIASγ, nuclear IKKγ is phophorylated by ATM and SUMOylated by PIASγ. This leads to cytoplasmic export of an IKKγ-ATM complex and subsequently activation of the IKK complex (Stilmann et al. 2009).

4.4.3.5 The non-canonical NF-κB pathway

The non-canonical NF-κB pathway can be stimulated by a distinct subset of stimuli, such as CD40L, lymphotoxin-β, B cell activating factor of the TNF family (BAFF), lipopolysaccharide (LPS) and latent membrane protein-1 (LMP1) of Epstein-Barr virus (Senftleben et al. 2001; Perkins 2007; Vallabhapurapu and Karin 2009). This stimulation leads to stabilization and autophosphorylation of the NF-κB inducing kinase (NIK), which activates an IKKα homodimer followed by the proteolytic processing of the NF-κB precursor p100 to p52 (Senftleben et al. 2001). The non-canonical NF-κB pathway is involved in the development of lymphoid organs and the adaptive immune system and it has been described in B cell development and survival (Senftleben et al. 2001; Nishikori 2005).

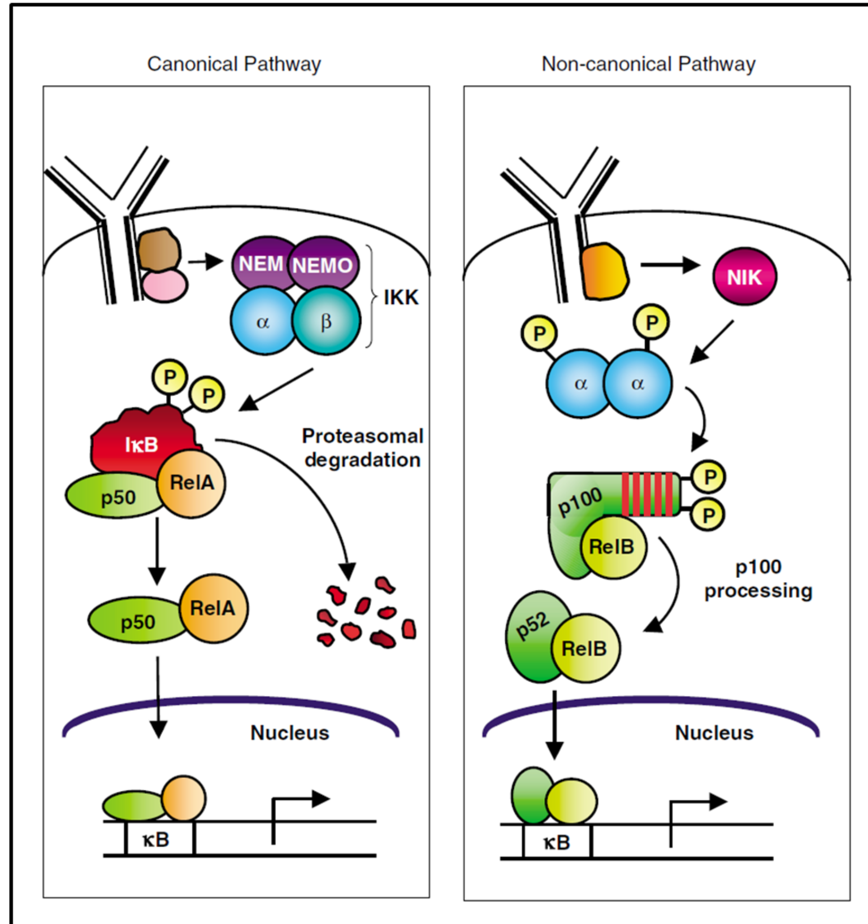


Figure 2: The canonical and non-canonical NF-κB pathways (modified from Gilmore 2006).

The canonical NF-κB pathway is regulated by an IKK complex consisting of the catalytic subunits IKKα and IKKβ and the regulatory subunit IKKγ (NEMO). The active IKK complex phosphorylates IκB and thus induces its degradation. As a consequence, the NF-κB subunits are freed and induce transcription of their target genes. Target genes include chemokines, cytokines and adhesion molecules implicated in inflammation and cell survival. The non-canonical NF-κB pathway is regulated by the NF-κB inducing kinase (NIK), which activates an IKKα homodimer followed by proteolytic processing of the NF-κB precursor. The non-canonical NF-κB pathway is involved in the development of lymphoid organs and the adaptive immune system.

4.4.3.6 Atypical NF-κB activation

The NF-κB pathway can also activated in atypical manner, where IκB is phosphorylated and degraded independent of the IKK complex. For example, some stimuli, such as treatment with nerve growth factor (NGF), hypoxia and reoxygenation or hydrogen-peroxide stimulation results in IκBα phosphorylation

at tyrosine Tyr-24, not at serine Ser-32 and Ser-36, and its subsequent degradation (Perkins 2007).

4.4.3.7 NF-κB activation in diseases

The NF-κB pathway has been described as a conductor of inflammatory processes in response to pathogens, stress signals or pro-inflammatory cytokines. Because most promoters of cytokines contain NF-κB binding sites, its activation results in expression of cytokines and chemokines, but also in expression of anti-microbial peptides, stress response proteins and anti-apoptotic proteins (Barnes and Karin 1997; Li et al. 2001; Smale 2011). Local production of cytokines and growth factors support cancer cell survival and invasive properties, but these effects alone would not suffice to generate oncogenic events. However, the production of reactive oxygen species (ROS) and metabolites of inflammation threaten DNA integrity, thus NF-κB provides a link between inflammation and cancer (Kim et al. 2006). NF-κB may promote oncogenic conversion of cells and it facilities oncogenesis at later stages (Baldwin 2001; Karin and Lin 2002). In accordance to this, many cancer cells exhibit a constitutive NF-κB activation (Gilmore et al. 2002).

Adding yet another level of complexity, NF-κB can become activated by chemotherapy or irradiation of cancer cells. This unwanted effect suppresses the pro-apoptotic potential of anti-cancer therapy and promotes chemoresistance (Nakanishi and Toi 2005; Gilmore 2006; Kim et al. 2006). Several anti-apoptotic factors are regulated by NF-κB, for example the anti-apoptotic proteins Bcl2, Bcl_{XL} and cIAP1/2, vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMP's), which in concert support angiogenesis and metastasis (Baldwin 2001; Karin and Lin 2002). It has been shown that inhibition of NF-κB can impair tumor cell growth and increase the susceptibility of tumor cells to anti-cancer treatment (Karin and Lin 2002; Kim et al. 2006; Schön et al. 2008; Amschler et al. 2010).

4.4.3.8 NF-κB in melanoma

The NF-κB pathway is constitutively activated in many melanoma cells, where it promotes survival and tumor progression through increased expression

of anti-apoptotic proteins, such as TNF receptor associated factor (TRAF) 1/2, survivin, anti-apoptotic members of the Bcl2-family and cell cycle regulatory proteins such as cyclin D1 and cyclin dependent kinase 2 (CDK2).In addition, it suppresses the TNF related apoptosis inducing ligand (TRAIL)-induced apoptosis (Shattuck-Brandt and Richmond 1997; Amiri and Richmond 2005). The tumor-promoting chemokines CXCL1 and CXCL8 are induced by NF-κB and, via a positive feedback loop, induce NF-κB activity, thus maintaining an increased activation level of NF-κB (Yang and Richmond 2001). Moreover, the NF-κB activity facilitates invasion and metastasis of melanoma cells by inducing expression of intercellular adhesion molecule-1 (ICAM-1, CD54), vascular adhesion molecule-1 (VCAM-1, CD106) and MMPs (Amiri and Richmond 2005). Furthermore, the increased expression of the ATP-binding cassette (ABC) transporter by NF-κB decreases the anti-apoptotic effect of chemotherapeutics (Amiri and Richmond 2005). Over-activation of the PI3K/AKT and the RAS/RAF/MEK/ERK pathways is thought to activate the IKK complex in melanoma cells, and this constitutive IKK activation then causes constitutive NF-κB activity (Dhawan et al. 2002; Amiri and Richmond 2005). Reflecting the impact of constitutive NF-κB activation, several studies have shown that inhibition of NF-κB can inhibit tumor growth, angiogenesis and metastasis in melanoma and increase the susceptibility to anti-cancer treatment (Huang et al. 2000; Amiri et al. 2004; Schön et al. 2008; Amschler et al. 2010; Pletz et al. 2012).

5 Aim of the study

Advanced melanomas are almost universally resistant to chemotherapeutic drugs. To overcome this chemoresistance is arguably the most important challenge in today´s melanoma therapy. The NF-κB pathway is relevant for drug resistance. Melanoma cells show enhanced endogenous NF-κB activity and NF-κB can be activated by some chemotherapeutics. Indeed, inhibition of the NF-κB pathway may help to overcome chemoresistance in melanoma cells.

Using cultured melanoma cells as model systems, the following questions should be addressed in this study:

1. Are there differences between human melanoma cell lines regarding their components of the IKK complex, IKKα and IKKβ, respectively?
2. Are there differences between various cytostatic drugs regarding NF-κB activation in melanoma cells?
3. Can overcoming drug-induced NF-κB activation contribute to the anti-tumoral activity of cytostatics?
4. Can selective targeting of components of the IKK complex affect drug-induced NF-κB activation? Do IKKα and IKKβ have different effects?
5. How does targeting IKKα or IKKβ, respectively, by specific small molecule inhibitors affect drug-induced NF-κB activation? Can such small-molecule inhibitors mimic the effects of targeting these IKK subunits by genetic approaches?
6. Is there evidence for a crosstalk with other molecules impacting on drug-induced NF-κB activation?

These questions should be approached by various methods reaching from biochemical and molecular biology techniques to experiments assessing cellular functions relevant for tumorigenesis and tumor progression. In order to better understand inducible chemoresistance, chemotherapeutic drugs established in the clinic were tested first for their ability to enhance NF-κB activity and the downstream gene transcription in melanoma cells. Once identified, this chemotherapeutic-induced NF-κB activity was analyzed following inhibition of either of the two major kinases of the NF-κB pathway, IKKα and IKKβ. Towards this end, specific small molecule compounds were investigated. While previous studies have suggested a considerable role of IKKβ inhibition for overcoming chemoresistance of melanoma cells, very little is known about IKKα in this respect. Two representative compounds, one directed against IKKα (BAY32-5915) and one against IKKβ (KINK-1), were used in this study. KINK-1 inhibits endogenous and stimulated NF-κB activity as described recently (Schön et al. 2008). The activity of BAY32-5915 (8-hydroxyquinoline-2-carboxylic acid) in melanoma cells was first investigated in this study. The experiments were paralleled and complemented by alternative inhibition strategies to confirm the target specificity of the approaches.

6 Material and Methods

6.1 Materials

Lab equipment	Company
Autoclave	Sanyo
See-saw rocker SSL4	Stuart
CO_2 incubator HeraCell 150i	Heraeus
Refrigerated centrifuge	Eppendorf
Horizontal electrophoresis System Sub-Cell®	Bio-Rad
Gradient thermocycler Mastercycler	Eppendorf
Microcentrifuge	Sprout
Magnetstirrer	VWR
Microscope Axiovert 200	Zeiss
Microscope Axioimager M1	Zeiss
Microscope Axioskop 2	Zeiss
Vertical electrophoresis System Mini-PROTEAN®TetraCell	Bio-Rad
Multichannel pipette	Brand
pH-meter FE20-Five easy™	Mettler Toledo
Pipette set: 1000, 100 and 10 µl	Eppendorf
Refrigerators: +4; -20; -80°C	Liebherr
Waterbath	Memmert
Microwave Micromat	AEG
Centrifuge Multifuge 15-R	Heraeus
Centrifuge Pico 17	Heraeus
Vortex L46	Labinco
Double distilled water system arium® 611VF	Sartorius
Cryo freezing container	Nalgene
GloMax® 96 Microplate Luminometer	Promega

Lab equipment	Company
Ultrasonic bath Sonorex RK 100	Bandelin
Rocking platform Polymax 1040	Heidolph
Spectrometer Genesys 10Bio	Thermo Scientific
Trans -Blot SD semi-dry transfer cell	BioRad
XCell SureLock™ Mini-Cell vertical electrophoresis system	Invitrogen
LAS-4000 imaging system	Fujifilm
Appliskan Multimode Microplate Reader	Thermo scientific
Gel imaging system U:Genius	Syngene
Sterile bench	Heraeus
Scale Vicon	Acculab
Micro scale ALC	Acculab
Thermal mixer ThermoStat plus	Eppendorf

Disposable and re-usable materials	Company
6-well plates	Greiner Bio One
12-well plates	Greiner Bio One
96-well plates flat bottom	Greiner Bio One
96-well plates luminescence	Greiner Bio One
PCR tubes	Biozym
Micro test tubes	Eppendorf
Cell count chamber Neubauer improved	Optik Labor
Cell culture flask 75cm^3	Greiner Bio One
Falcon tubes 15 ml; 50 ml	Greiner Bio One
Chromatography paper Whatman™	GE Healthcare
Freezing tubes Cryo.S	Greiner Bio One
Pipette tips 10, 200, 1000 µl	Starlab

Disposable and re-usable materials	Company
Syringe 1 ml Omnifix 40 solo	B. Braun
Needle Micolance 3	BD
Filter pipette tips for PCR epT.I.P.S.®	Eppendorf
Filter pipette tips for RNA TipOne	Starlab
Gloves	Meditrade
One way pipetts 5, 10, 25 ml	Sarstedt
Culture-Inserts	IBIDI
8-well CultureSlide	BD

Cell Culture Media	Company
FCS Gold Eu approved	PAA
DMEM high glucose (4.5 g/l)	PAA
RPMI 1640 with L-Glutamine	PAA
Penicillin/Streptomycin (100x)	PAA
L-Glutamin (200mM)	PAA
Trypsin-EDTA (1:250) (1x)	PAA
Freezing Medium Cryo-SFM	Promocell
Trypan Blue solution 0.4 %	Sigma
Dulbecco's PBS (1x) without Ca & Mg	PAA

Kits	Company
Nuclear extraction kit	Panomics
Cell Death Detection ELISA^{PLUS}	Roche
Luciferase assay	Promega
E.Z.N.A. RNA isolation Kit	Omega
First Strand cDNA Kit	Fermentas

Kits	Company
CellTiter 96® Non-Radioactive Cell Proliferation Assay	Promega

Reagents/Chemicals	Company
Chemotherapeutics	
Temozolomide	AG Emmert
Cisplatin	Sigma
Doxorubicin	Sigma
Vincristine	Sigma
5'fluorouracil	Sigma
RT-PCR	
LE Agarose	Biozym
GelRed Nucleic Acid Gel Stain	Biotium
Taq DNA Polymerase	peqlab
dNTP set	Fermentas
Western blot	
DMSO	Sigma-Aldrich®
EDTA disodium salt dihydrate	Carl Roth®
Tris(hydroxymethyl)aminomethane	Merck Millipore
Sodium n-Dodecyl Sulfate (SDS)	Merck Millipore
β-mercaptoethanol	Carl Roth®
Complete, Mini Protease Inhibitor Cocktail Tablets	Roche Applied Science
Glycerol	Merk Millipore
Bromophenol Blue sodium salt	Sigma-Aldrich®
Glycine	Merk Millipore
Methanol	Merk Millipore
Ponceau-S	Sigma-Aldrich®

Reagents/Chemicals	Company
Acetic acid (glacial) 100%	Merk Millipore
Sodium chloride (NaCl)	Merk Millipore
Tween® 20	Carl Roth®
Albumin from bovine serum (BSA)	Sigma-Aldrich®
4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI)	Sigma-Aldrich®
BSA standard Set, ready-to-use	Fermentas
Bradford solution 5 x	Bio-Rad
Ammonium peroxodisulfate (APS)	Merk Millipore
N,N,N',N'-Tetramethyl ethylenediamine (TEMED)	Merck
30 % Acrylamide/Bis Solution	Bio-Rad
Nitrocellulose membrane	Bio-Rad
Methanol	Merck millipore
Hydrogen peroxide (H_2O_2)	Merck millipore
Luminol	Sigma-Aldrich®
p-Coumarine acid	Sigma-Aldrich®
PageRuler™ Prestained Protein Ladder	Fermentas
Transfection	
Lipofectamin™ 2000	Invitrogene
Immunofluorescence	
Triton® X-100	Merk Millipore

Software	Company
Multi Gauge	Fujifilm
MetaMorph® Microscopy Automation & Image Analysis Software	Molecular Devices
AxioVision Software	Zeiss
ImageJ	Freeware

Software	Company
Microsoft Office Exel	Microsoft

6.1.2 Solutions and buffers

6.1.2.1 Cell culture

DMEM complete

- DMEM
- 10 % (v/v) FCS
- 1 % (v/v) Penicillin/Streptomycin
- 1 % L-Glutamine

RPMI complete

- RPMI
- 10 % (v/v) FCS
- 1 % (v/v) Penicillin/Streptomycin
- 1 % L-Glutamine

6.1.2.2 Western blot

SDS-lysis buffer

- 50 mM Tris pH 7.0
- 1 % SDS
- 5 % β-mercaptoethanol
- 1 x Complete Mini (Roche)

5 x SDS sample buffer

- 200 mM Tris pH 6.8
- 4 % SDS
- 40 % glycerol
- 0.07 % bromophenol blue

10 x Running buffer

- 1.92 M glycine
- 250 mM Tris
- 5 % SDS

Transfer buffer

- 48 mM Tris
- 39 mM glycine
- 0.0375 % SDS
- 20 % methanol

Ponceau-S

- 0.1 % Ponceau-S
- 5 % acetic acid

TBST

- 10 mM Tris
- 1 mM EDTA pH 8.0
- 150 mM NaCl
- 0.05 % Tween 20

BSA/TBST

- 5 %BSA in TBST

ECL-solution

Solution 1

- 0,1 M Tris pH 8.5
- 0.4 mM p-cumaric acid
- 1.125 mM luminol

Solution 2

- 0.1 M Tris pH 8.5
- 0.005 % H_2O_2

Solution 1 and 2 have to mix 1:1 freshly before using.

6.1.2.3 Immunofluorescence

Triton/BSA

- 0.5 % Triton X-100
- 0.2 % BSA

in PBS

6.2 Methods

6.2.1 Cell culture

The human melanoma cell lines and the modified human cervical carcinoma cell line HeLa-mCD40L-hBAFF were cultivated in DMEM complete and the B lymphoma cell line SU-DHL-4 was cultivated in RPMI complete at 37°C in a humidified atmosphere with 5 % CO_2.

For passaging the suspension cell line SU-DHL-4 the cells were harvested by a centrifugation step at 850 x g for 10 min at room temperature; subsequently the cells were re-suspended in 10 ml RPMI complete and 2 ml of the cell suspension were transferred to a new culture flask with fresh media for further cultivation. In contrast, the adherent cells were washed with PBS followed by trypsin/EDTA treatment for approximately 2 minutes at 37°C for detaching the cells from the wall of the culture flask. When cells were detached the enzymatic activity of trypsin/EDTA was stopped by addition 10 ml of DMEM complete. Similarly to suspension cells, 2 ml of cell suspension were used for further cultivation.

When cells were harvested for analysis the culture dishes were washed once with cold PBS and then the cells were scratched off in 1 ml cold PBS and transferred to a reaction tube. Subsequently, the cells were precipitated by a centrifugation step at 850 x g for 10 minutes at 4°C. The supernatant was discarded and the cell pellet was immediately used for analysis or stored at -20°C.

For cryopreservation the number of cells was determined in a cell counting chamber; therefore the cell suspension was mixed 1:10 with trypan blue solution. Subsequently, the cells were harvested by a centrifugation step at 850 x g for 10 minutes at room temperature and re-suspended in freezing medium to get a maximum concentration of 5 x 10^6 cells/ml. This freezing medium - cell suspension was transferred in specific freezing tubes and slowly cooled down in a cryo freezing container in a -80°C freezer over night. Next day, the cryotubes were transferred and stored in liquid nitrogene (-176 °C). For re-cultivation the cells were thawed and subsequently transferred to 37°C preheated cell culture media; the media was changed after 24 hours and 24 hours later cells were used for experiments.

All steps of cell culture procedure were done in a clean bench with sterilized equipments.

6.2.2 Protein extracts

6.2.2.1 Whole cell extract

To prepare whole cell lysates, cells were harvested and re-suspended in 50 - 300 µl SDS-lysis buffer, depending on cell pellet size, and were heated at 95°C for 5 minutes. The extracts were homogenized in ultrasonic bath for 5 minutes. After centrifugation at 25.000 x g for 10 minutes at 4°C the supernatant was transferred to a new reaction tube, this was the whole cell extract. Immediately, the protein concentration was determined and protein extracts were stored at -80°C. During, the whole procedure the samples were stored on ice.

6.2.2.2 Cytoplasmic and nuclear extracts

To separate cytoplasmic and nuclear extracts the Panomics nuclear extraction kit was used. For each preparation the provided buffers were freshly mixed with DTT, protease and phosphatase inhibitors. The harvested cells were re-suspended in 70 - 150 µl buffer A working reagent followed by a 10 minutes incubation on a rocking platform in a ice bucket. After centrifugation at 14,000 x g for 3 minutes the supernatant was transferred to a new reaction tube, this is the cytoplasmic fraction. The remaining pellet was covered with 25 - 50 µl of the buffer B working reagent; vortexed at highest settings for 10 seconds and incubated on ice for 1 hour. The sample tubes were shaken every 20 minutes by hand and subsequently centrifuged at 14,000 x g for 5 minutes at 4°C. The supernatant, the nuclear extract, was transferred in a new reaction tube. The protein concentration was immediately determined and extracts were stored at -80°C.

6.2.3 Determination of protein concentration

Protein concentration was determined by the Bradford method. Therefore, 1 µl of the protein extracts or 1 µl extraction buffer and the BSA standards was diluted in 800 µl H_2O and then mixed with 200 µl Bradford reagent. The protein concentration was calculated by measuring the absorbance at 595 nm with the Genesys 10 Bio spectrometer.

6.2.4 Western blot

6.2.4.1 SDS-PAGE and semi-dry Western blot of proteins between 15 to 120 kDa

To analyze the cellular synthesis of proteins the protein extracts were mixed with H_2O and 5 x SDS sample buffer to get a similar concentration of each sample; after heating at 95°C for 5 minutes 30 µg protein were loaded for SDS-polyacrylamide gel electrophoresis (PAGE). The polyacrylamide concentration of the gels depended on the size of the protein of interest. The

electrophoresis was performed at 40 mA per gel until the blue dye of the sample buffer reached the end of the polyacrylamide gel.

The transfer of proteins onto a nitrocellulose membrane was performed with the semi-dry system from BioRad. Therefore, filter paper and membrane were soaked in cold transfer buffer and “sandwich” was built between the anode and cathode, whereby the proteins run in the electric field toward the anode and out of the gel onto the membrane. The transfer occurred at 150 mA for 55 minutes and its success was proved by Ponceau S staining of the membrane.

6.2.4.2 SDS-PAGE and semi-dry Western Blot of proteins larger than 120 kDa

To clearly separate and transfer proteins larger than 120 kDa, precasted *NuPage®* tris-acetate gels by Invitrogen and the related electrophoresis chamber, blot module, precast sample, running and transfer buffer were used. According to manufacturer information, the running conditions of protein electrophoresis were 150 V for 1 hour and of protein transfer 30 V constant for 1 hour.

6.2.4.3 Immunodetection

Single proteins on the membrane were detected by specific antibodies. Therefore, the membrane was blocked for 1 hour in 5 % BSA/TBST followed by antibody exposition over night at 4°C. Next day, the membrane was washed in TBST and incubated with horseradish peroxidase (HRP)- conjugated secondary antibody for 1 hour. After an additional wash step the membrane was incubated with ECL solution to detect the HRP enzyme activity. The resulting luminescence was detected by the LAS-4000 imaging system.

Primarily antibody	Company
Anti-Bcl-xL (54H6)	Cell Signaling Technology®
Anti-IKKα	Cell Signaling Technology®
Anti-Actin, clone C4	Merk Millipore
Anti-NF-κB2 p100/p52 (18D10)	Cell Signaling Technology®
Anti-IKKβ (2C8)	Cell Signaling Technology®
Anti-Lamin A/C	Cell Signaling Technology®
Anti-GAPDH (14C10)	Cell Signaling Technology®
Anti-Cyclin D1 (DCS6)	Cell Signaling Technology®
Anti-Survivin (D-8)	Santa Cruz Biotechnology, Inc.
Anti-β-Catenin (14)	BD Bioscience
Anti-Bcl-2 (C-2)	Santa Cruz Biotechnology, Inc.
Anti-Bax (2D2)	Santa Cruz Biotechnology, Inc.
Anti-ATM (D2E2)	Cell Signaling Technology®
Phospho-ATM (Ser1981) (10H11.E12)	Cell Signaling Technology®
Anti-phospho-H2A.X (Ser139)	Merk Millipore
Primarily antibody	Company
Anti-Bcl-xL (54H6)	Cell Signaling Technology®

Secondary antibody	Company
Anti-Mouse IgG (H+L), HRP Conjugate	Promega
Anti-Rabbit IgG (H+L), HRP Conjugate	Promega
Alexa Fluor® 555 Goat Anti-Rabbit IgG (H+L)	Invitrogene
Anti-mouse IgG (H+L), F(ab')2 Fragmen (Alexa Fluor® 488 Conjugate)	Cell Signaling Technology®

6.2.5 Apoptosis assay

The apoptosis rate was determined by the Cell Death Detection ELISA^{Plus} Kit measuring the generation of histone-bound DNA fragments which are enriched in the cytoplasm of apoptotic cells. For apoptosis analysis melanoma cells were seeded at a concentration of 1 x 10^5 cells/ml on a 96-well plate and cultivated over night to become adherent. Afterwards, melanoma cells were treated with chemotherapeutics or inhibitors. Then the ELISA was performed according to manufacturers manual; thereby cells were lysed and the cytoplasm fraction was mixed with antibodies against histone and DNA. The histone antibody is biotin-labeled and bound to the streptavidin coated plate and the HRP-label of the DNA antibody catalyzed the substrate reaction. This reaction resulted in a color change and was measured by wavelength of 405 nm in the plate reader. The values of the absorbance at 405 nm were normalized to untreated cells to determine the apoptosis rate.

6.2.6 Luciferase assay

Melanoma cells were seeded in 12-well plates at 1 x10^5 cells/ml. After 24 hours cells were transiently transfected by lipofectamin 2000 (2 µl/ml) with 0.5 µg/ml of the pGL4.32[*luc*2P/NF-κB-RE/Hygro] Vector (Promega), containing five copies of an NF-κB response element that drives the transcription of the luciferase gene luc2P, and with 0.1 µg/ml of the pRL-TK vector as an internal control. The pRL-TK vector (Promega) contained cDNA encoding *Renilla* luciferase under the control of the herpes simplex virus-thymidine kinase (HSV-TK)- promoter. 24 hours after transfection cells were treated by chemotherapeutics and inhibitors for indicated times and subsequently luciferase assay was performed. Therefore, 30 µl of the cell lysate were transferred to a non-transperent 96-well plate and the chemiluminescence was measured in the GloMax® 96 Microplate Luminometer. First, 80 µl of the luciferase substrate were add to the cell lysate and the luminescence was detected, this reaction was stopped by the addition of 80 µl of the *Renilla* luciferase substrate. The ratio of the luciferase and the *Renilla* luciferase

chemiluminescence reflected the NF-κB activity and the values were normalized to untreated cells.

6.2.7 RNA isolation

For RNA isolation 1 x10^5 cells/ml were seeded in 6-well plate for 24 hours, treated with chemotherapeutics and inhibitors and harvested. The RNA was isolated with the E.Z.N.A.® Total RNA Kit according to manufacturers protocol. The cells were disrupted with lysis buffer and homogenized by syringe and needle; after addition of 70 % ethanol the mixture was transferred to the HiBind RNA spin column washed 3 times by centrifugation at 10,000 x g for 60 seconds at room temperature. Subsequently, the column was dried by centrifugation and RNA was eluted from column by adding 30 µl DEPC-treated water. Afterwards, the amount of RNA was calculated by measuring the absorbance at 260 nm with the Genesys 10 Bio spectrometer.

6.2.8 Reverse transcription-polymerase chain reaction

For reverse transcription-polymerase chain reaction (RT-PCR) analysis 2 µg of the total RNA was transcribed to complementary DNA (cDNA) with the First Strand cDNA Synthesis Kit containing the recombinant moloney murine leukemia virus (M-MuLV)- reverse transcriptase. Exclusively, messenger RNA (mRNA) was transcribed in cDNA by using oligo(dT)$_{18}$ primer which bind the poly(A) tail of the mRNA. Subsequently, the cDNA was analyzed for transcripts of genes of interest by PCR using specific designed primers.

General PCR components

Primer forward	1 µl (0.4 µM)
Primer revers	1 µl (0.4 µM)
10 x reaction buffer	2.5 µl
dNTP's	1 µl (0.4 mM)
Taq polymerase	0.2 µl (1Unit)
ddH$_2$O	add 24 µl
cDNA	1 µl

General cyclin parameters

1	Initial DNA denaturation	94°C	3 min
2	Denaturation	94°C	30 sec
3	Primer annealing	variable	30 sec
4	Extension	72°C	30 sec
5	Numbers of cycles (step 2 -4)	26	
6	Finale extension	72°C	10 min

Target gene	Primer	Anneal. temp.
GAPDH for	5' – CTT TGG TAT CGT GGA AGG ACT C – 3'	58°C
GAPDH rev	5' - TTC GTT GTC ATA CCA GGA AAT G - 3'	
IL-6 for	5'-AGCTATGAACTCCTTCTCCAC-3'	60°C
IL-6 rev	5'-AGCATCCATCTTTTTCAGCC-3'	
CXCL8 for	5' - ACA AGC TTC TAG GAC AAG AGC CAG GAA G - 3'	62°C
CXCL8 rev	5' - GTG AAT TCA GTG TGG TCC ACT CTC AAT C - 3'	
CXCL1 for	5' - ATG GCC CGC GCT GCT CTC TCC -3'	58°C

Target gene	Primer	Anneal. temp.
CXCL1 rev	5' - CTT AAC TAT GGG GGA TGC AGG - 3'	
CCL2 for	5' - GCT CAT AGC AGC CAC CTT CAT TC- 3'	58°C
CCL2 rev	5' - TGC AGA TTC TTG GGT TGT GGA G - 3'	
CCND1 for	5'-AAC AGA AGT GCG AGG AGG AG-3'	52°C
CCND1 rev	5'-AGG GCG GAT TGG AAA TGA AC-3'	
ICAM1 for	5' - TGA CCA GCC CAA GTT GTT GG - 3'	55°C
ICAM1 rev	5' - ATC TCT CCT CAC CAG CAC CG - 3'	
SERPINB5 for	5'-TTT CAG ACT CAA CAA GAC AGA C-3'	52°C
SERPINB5 rev	5'-CCT AGA TTT TCC AGA CAA GCC-3'	
CHUK for	5'-GCA GGG AAA AAG GCA GAA AG-3'	54°C
CHUK rev	5'-GGA GTT ACC ACA CAT GAC AG-3'	

6.2.9 Migration assay

The migration ability of melanoma cells was examined by migration assay using two chambers Ibidi culture-inserts. In each chamber 70 µl of 1 x 10^5 cells/ml cell suspension were seeded and cultured over night to become adherent. Next day, the culture-insert was removed and the dish was washed with DMEM complete; afterwards the cells were treated with inhibitors and photo documented after 0, 24, 48 and 72 hours.

6.2.10 Transfection of siRNA

To reduce the protein level of IKKα and IKKβ the cells were transfected with siRNA against these proteins. The siRNA was purchased by QIAGEN (see below). To reduce the protein level 1 x 10^5 cells/ml in DMEM w/o Pen/strep and the lipofectamin 2000 (3 µl/ml)- siRNA mixture were seeded in 6-well plates for 48 hours. For the effective reduction of IKKβ a second siRNA transfection for further 24 hours was necessary. Then cells were harvested for Western blot or

RT-PCR analysis or previously treated with chemotherapeutics. When NF-κB activity by luciferase assay should be examined in cells with reduced IKKα or IKKβ level, the lipofectamin 2000-siRNA mixture was extended by vectors of the luciferase assay. Furthermore, when siRNA treated cells should be analyzed for apoptosis or proliferation rate, 100 µl of the melanoma cell-lipofectamin 2000-siRNA mixture were seeded per well of a 96-well plate for 48 hours; followed by chemotherapeutic exposition and assay.

siRNA against IKKα: Hs_CHUK_8 FlexiTube siRNA

siRNA against IKKβ: Hs_IKKB2_1 FlexiTube siRNA

6.2.11 Immunofluorescence

For immunofluorescence staining the cells were seeded in 8-well culture slide and exposed to chemotherapeutics for different times. Afterwards, the cells were fixed by -20°C methanol exposition for 7 minutes at -20°C. When the slide was air dried, the fixed cells were incubated in triton/BSA to permeabilize the cell membrane and block unspecific binding sites. Afterwards, slides were probed with primary antibody for 1 hour at room temperature followed by washing with PBS before incubating with fluorophore-conjugated secondary antibody and DAPI for 1 hour. Further, cells were washed with PBS again and immediately covered with Fluoromount™ mounting medium and a coverslip. When Fluoromount™ was dried the immunofluorescence signals were detected and documented with *Axioimager M1* microscope and *AxioVision* software.

6.2.12 Proliferation assay

To determine the proliferation rate the CellTiter 96® Non-Radioactive Cell Proliferation Assay was used. Therefore, 1 x 10^5 cells/ml were seeded and treated in 96-well plates. The assay was performed by adding 15 µl of the premixed Dye solution to the wells and cultured the cells for further 4 hours at 37°C in a humidified, 5 % CO_2 atmosphere; during this time the dye is converted by living cells. By adding 100 µl of the stop solution the conversion of

the dye was stopped and the products were stabilized. The plate was stored in 37°C incubator over night to solve the dye crystals and then the absorbance at 570 nm and 630 nm was recorded by *Appliskan* plate reader. The absorbance at 570 nm was proportional to the number of living cells and the absorbance at 630 nm was used as reference wavelength to reduce the background. The proliferation rate was calculated by the difference of absorbance values of both wavelengths and the calculated values were normalized to untreated cells.

7 Results

7.1 The levels of IKK subunits vary between melanoma cell lines

The NF-κB pathway is constitutively active in melanoma cells (Shattuck-Brandt and Richmond 1997; Amiri and Richmond 2005), although the level of activation may differ (Amschler et al. 2010). The canonical NF-κB pathway is activated by the IKK complex consisting of three subunits: IKKα and IKKβ, the catalytic subunits and IKKγ/NEMO the regulatory subunit. In order to assess whether there are differences regarding the catalytic subunits of the IKK complex, the expression IKKα and IKKβ, respectively, was determined by Western blot analysis of whole cell lysates prepared from selected melanoma cell lines generated from cutaneous metastatic tumors (A375, MMNH and MV3) or a lymph node metastasis (LOX). It was found that the cell lines showed considerably different expression of IKKα and IKKβ (Figure 3). Expression of IKKα was highest in the cell lines A375 and MMNH, whereas MV3 showed intermediate levels and LOX showed the lowest level of IKKα. Similarly, the highest expression of IKKβ was shown in the MMNH cell line; intermediate expression was detected in A375 and MV3 cell lines and the lowest IKKβ level was shown in LOX cells. However, given that MMNH and MV3 have higher endogenous NF-κB activity than A375 and LOX (Amschler et al. 2010), the correlation between NF-κB activity and expression levels of the catalytic subunits in the four melanoma lines appeared to be limited.

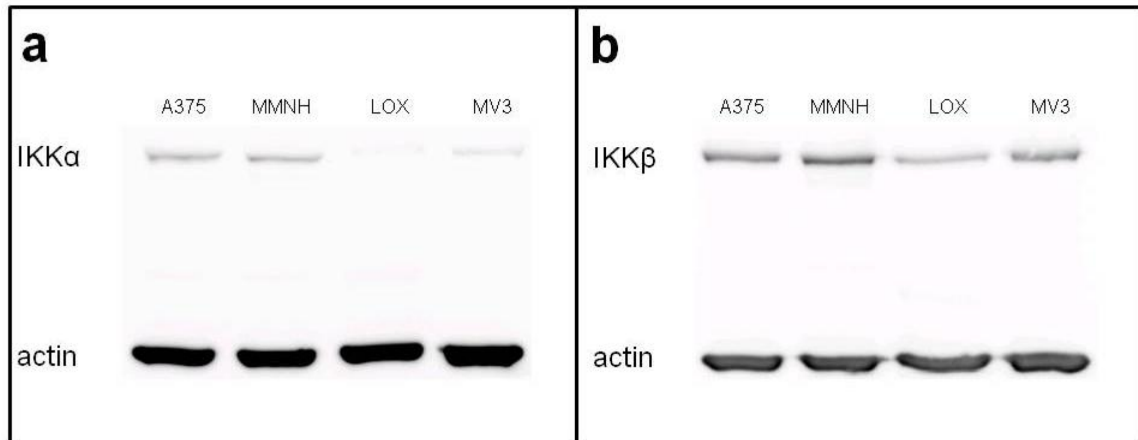


Figure 3: Differential expression of IKKα and IKKβ in melanoma cell lines.

Whole cell lysates of unstimulated melanoma cell lines were analyzed by Western blot for IKKα (a) and IKKβ (b). Additionally, the amount of actin was detected as internal loading control. These blots are representative for three independent experiments.

7.2 NF-κB activity and downstream gene transcription in human melanoma cells are induced by doxorubicin, but not by several other chemotherapeutics

Melanoma cells are highly resistant to chemotherapeutic treatment (Grossman and Altieri 2001). Additionally, some chemotherapeutic drugs may even activate mechanisms of resistance, such as NF-κB activity, in tumor cells (Das and White 1997; Wang et al. 1999). Based on this notion, several chemotherapeutic drugs with different modes of action were tested for their ability to induce NF-κB activity and apoptosis in melanoma cells (Figure 4):

Temozolomide is an alkylating agent that forms O^6-methylguanine. The generation of single - and double strand breaks activates apoptotic pathways (Agarwala and Kirkwood 2000). Cisplatin interacts with purine bases to form DNA-protein and DNA-DNA intra- and interstrand crosslinks, mainly intrastrand adducts. The formation of these adducts affects DNA replication, but not DNA synthesis, and apoptosis is induced by activating different signaling pathways (Siddik 2003). Doxorubicin belongs to the anthracyclines; these anti-cancer

drugs exert several mechanism of action: 1) DNA intercalation, 2) generation of free radicals, 3) binding and alkylation of DNA, 4) DNA cross-linking, 5) interference with DNA unwinding, strand separation and helicase activity, 6) membrane effects and 7) inhibition of topoisomerase II (Minotti et al. 2004). The vinca alkaloid, vincristine, inhibits the construction of the mitotic spindle by depolymerizing microtubules and blocking cell division (Jordan and Wilson 2004). Finally, 5-fluorouracil is an analogue of uracil and belongs to the group of anti-metabolites. Intracellularly, it becomes converted into several active metabolites which incorporate into RNA and DNA and inhibit the thymidylate synthase (Longley et al. 2003).

Human melanoma cells were exposed to all of these chemotherapeutics to assess NF-κB activity and apoptosis; different concentrations of each compound were tested in these experiments. For the NF-κB driven luciferase assay, melanoma cells were transfected with the required plasmids and the chemotherapeutic treatment followed for 8 hours on the next day. Subsequently, the NF-κB activity was determined by luciferase assay. Induction of apoptosis in melanoma cells was measured using the Cell death detection ELISA 24 hours after exposure to the selected chemotherapeutics. It was found that all chemotherapeutics increased apoptosis in melanoma cells; but only doxorubicin simultaneously stimulated NF-κB activity. In A375 cells, treatment with doxorubicin at a concentration of 1 μM increased the NF-κB activity 3.9-fold and the apoptosis rate 3.8-fold (Figure 4a). The NF-κB stimulation in LOX cells was only 1.5-fold at a concentration of 0.5 μM doxorubicin, whereas apoptosis was increased 5.7-fold compared to untreated cells (Figure 4b).

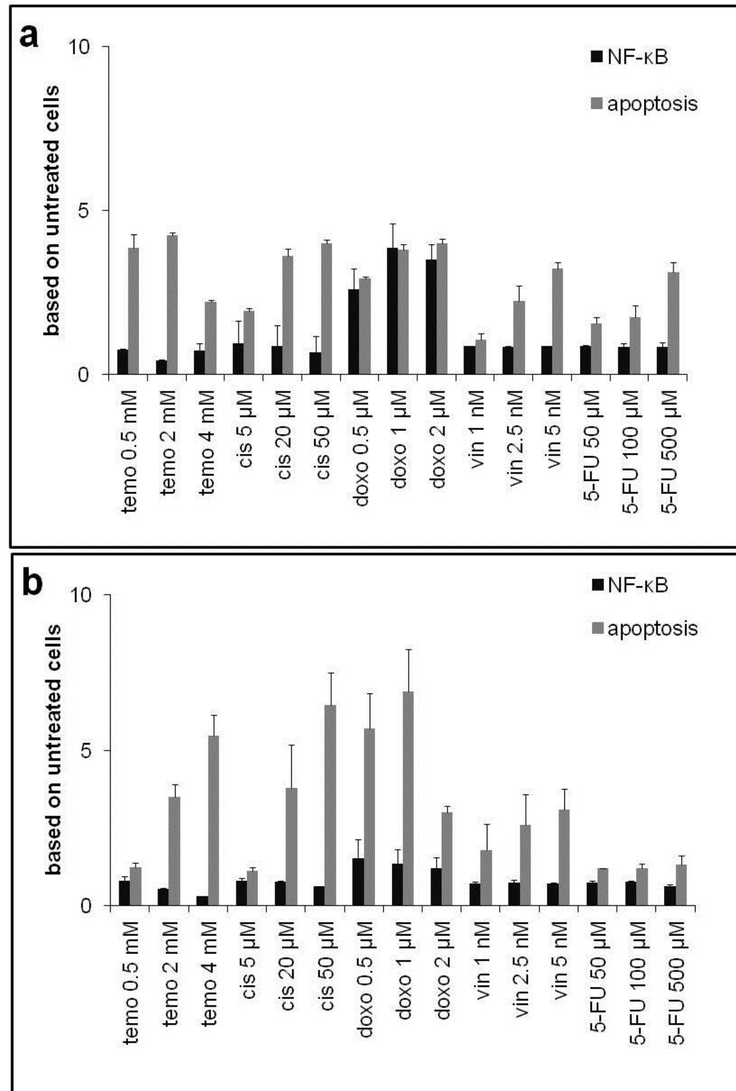


Figure 4: Doxorubicin, but not several other chemotherapeutic drugs, induces NF-κB activity in melanoma cells.

The melanoma cell lines A375 (a) and LOX (b) were exposed to various chemotherapeutic drugs at concentrations relevant in the clinic (individual concentrations indicated in the panels). The chemotherapeutics selected exert different modes of action (temo = temozolomide, cis = cisplatin, doxo = doxorubicin, vin = vincristine, 5-FU = 5-fluorouracil). While all drugs increased apoptosis in melanoma cells, only doxorubicin induced NF-κB activity in both cell lines (up to 3.9- fold in A375 and 1.5-fold in LOX). Prior to the chemotherapeutic treatment, melanoma cells were transfected with plasmids for the NF-κB-driven luciferase assay. NF-κB activity was measured 8 hours after treatment, and apoptosis was determined 24 hours after exposure to the compounds using an ELISA measuring the generation of histone-bound DNA fragments. a and b display the alteration of NF-κB activity (black bars) and apoptosis rate (grey bars) caused by the chemotherapeutic treatment compared to vehicle treated cells. The values shown represent the average of two independent experiments, each performed in triplicate (±SEM).

To assess doxorubicin-induced NF-κB activation in more detail, A375 and LOX melanoma cells were exposed to varying concentrations of doxorubicin for 8 hours. NF-κB activity was determined using an NF-κB-dependent luciferase assay. Overall, doxorubicin-induced activation in LOX cells was somewhat weaker as compared to A375 cells (Figure 5). In A375 melanoma cells, treatment with 1 µM doxorubicin induced the highest NF-κB activation (3.4-fold) while in LOX cells the strongest induction of NF-κB activity was detected at 0.5 µM doxorubicin (2-fold). Interestingly, the doxorubicin concentration leading to the strongest NF-κB activation (i.e., 0.5 to 1.0 µM) corresponded to the doxorubicin concentration measured in the serum of doxorubicin-treated patients (Palm et al. 2001).

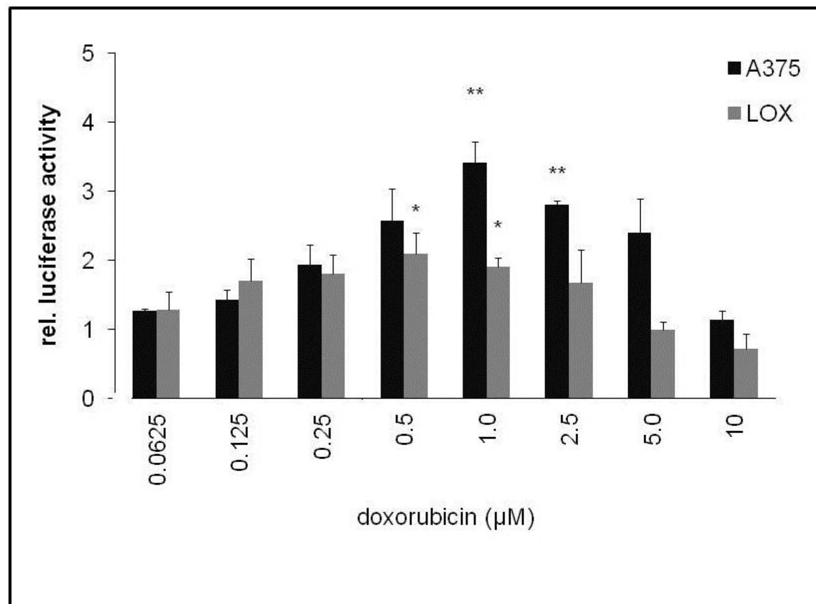


Figure 5: Doxorubicin-induced NF-κB activity is concentration dependent.

Two melanoma cell lines, A375 and LOX, were transfected with specific luciferase plasmids and incubated with different concentrations of doxorubicin for 8 hours on the next day. Doxorubicin-induced NF-κB activity was normalized to vehicle-treated cells. The strongest induction of NF-κB was detected when 1 µM doxorubicin in A375 cells (black bars) or 0.5 µM doxorubicin in LOX cells (gray bars) was used. The values display the average of three independent experiments, each performed in triplicate (±SEM). * = $p < 0.02$; ** = $p < 0.00005$ compared to vehicle-treated cells.

In order to analyze downstream effects of drug-induced changes of NF-κB activity, transcription of NF-κB regulated genes, which are thought to be involved in tumor progression, was assessed by semi-quantitative RT-PCR in melanoma cells. Enhanced transcription and synthesis of interleukin 6 (IL-6), interleukin 8 (CXCL8), CXCL1 (GROα) and CCL2 (monocyte chemotactic protein 1; MCP-1) correlate with progression and aggressiveness of melanoma. IL-6 is an NF-κB regulated inflammatory cytokine, which is expressed by human melanomas. High levels of IL-6 correlate with the metastatic properties of melanoma cells and chemoresistance in melanoma patients (Ilkovitch and Lopez 2008; Richmond et al. 2009). The expression of CXCL8 increases during melanoma progression and is regulated, at least in part, through NF-κB (Payne and Cornelius 2002). CXCL8 promotes growth, angiogenesis and metastasis of melanoma (Varney et al. 2006; Gebhardt et al. 2007). Likewise, NF-κB is also involved in the regulation of CXCL1, which is upregulated in human melanoma and has an important role in angiogenesis (Richmond et al. 2009). Further, CCL2, a chemokine that stimulates tumor vascularization, has been found highly expressed in melanoma cells but not in melanocytes (Graves et al. 1992). Recently, it was shown that chemotherapy-induced NF-κB activity controls transcription and secretion of *CCL2* and, thus, generates a pro-invasive microenvironment (Ohanna et al. 2011).

In A375 melanoma cells, the transcription of genes, which encode for IL-6, CXCL8, CXCL1 and CCL2, respectively, was markedly enhanced by treatment with doxorubicin for 4 hours (Figure 6). In accordance with the results of the luciferase assay, the treatment with 1 µM doxorubicin showed the strongest transcription increment of these genes (Figure 6).

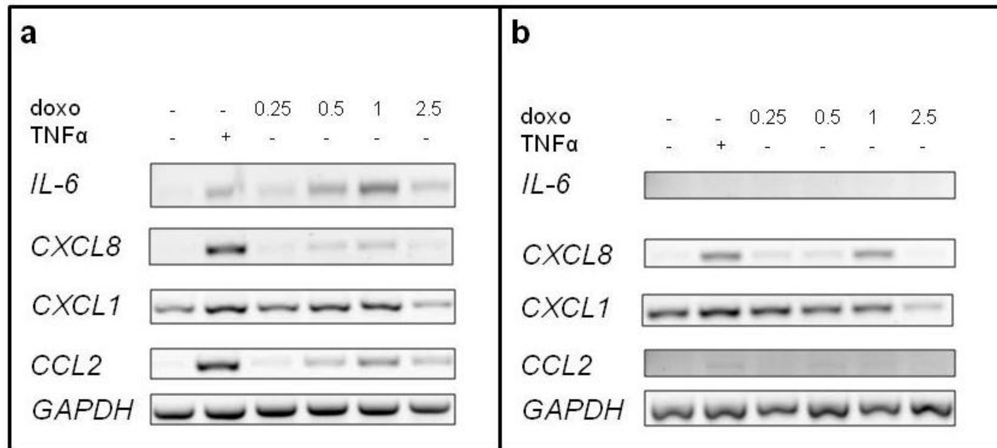


Figure 6: Doxorubicin induces transcription of NF-κB-regulated genes in a dose dependent manner.

Transcription of NF-κB-dependent genes was assessed using semi-quantitative RT-PCR in A375 (a) and LOX (b) melanoma cells exposed to different concentrations of doxorubicin for 4 hours. TNFα stimulated melanoma cells (20 ng rhTNFα / ml for 2 hours) were used as positive control. The exposure to 1 µM doxorubicin induced the strongest transcription of NF-κB-regulated genes. This experiment is representative for two independent experiments showing similar results.

In LOX cells, only the transcription of *CXCL8* was enhanced by doxorubicin, with a maximum at a concentration of 1 µM (Figure 6b). No induction of *IL-6* or *CCL2* was detected by semi-quantitative RT-PCR, and the transcription of *CXCL1* was not increased by doxorubicin treatment (Figure 6b).

To assess whether exposure to cytostatics, which did not activate NF-κB, affected the transcription on NF-κB regulated genes in melanoma cells, RT-PCR was performed following treatment with cisplatin. In contrast to doxorubicin, cisplatin did not enhance the transcription of *IL-6*, *CXCL8*, *CXCL1* or *CCL2*, respectively (Figure 7).

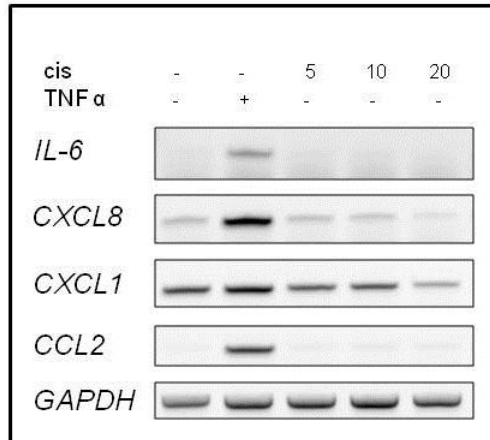


Figure 7: Cisplatin did not induce the transcription of NF-κB-regulated genes.

RT-PCR in A375 melanoma cells was performed as outlined in figure 6, except that the cells were incubated with cisplatin at the indicated concentrations (in µM). In parallel to the earlier observation that cisplatin did not activate NF-κB, no clear increase of NF-κB-dependent gene transcription could be detected. This experiment was repeated twice with similar results.

These results indicated that NF-κB activity and downstream gene products, which are thought to be involved in melanoma progression, are induced by doxorubicin. Considering the protective function(s) of NF-κB in tumor cells, it is reasonable to assume that the activation of NF-κB by doxorubicin represents a mechanism of inducible chemoresistance.

7.3 Doxorubicin induced NF-κB activity can be abrogated by an IKKβ inhibitor, but not by an IKKα inhibitor

Inhibition of NF-κB may increase the susceptibility of cancer cells to chemotherapeutic treatment (Huang et al. 2000; Amiri et al. 2004; Schön et al. 2008; Amschler et al. 2010; Pletz et al. 2012). Approaches taken to demonstrate that blocking NF-κB functions improved anti-tumoral therapies included the use of IKKβ inhibition or IKKα/IKKβ inhibitors, proteasome inhibition or IκBα dephosphorylation (Amiri et al. 2004; Schön et al. 2008; Yang

et al. 2009; Amschler et al. 2010). However, no reliable inhibitor for IKKα has been described yet.

Through collaboration with Dr. Ziegelbauer at Bayer HealthCare (Wuppertal), we were able to obtain the small molecule substance 8-hydroxyquinoline-2-carboxylic acid (BAY32-5915), which specifically inhibited IKKα kinase activity in cell free *in vitro* kinase assays using more than 50 recombinant enzymes. This novel compound potently and selectively inhibited IKKα at a LD_{50} from 60 nM (Figure 36).

In the first experiments, the ability of the IKKα inhibitor to inhibit TNFα-induced NF-κB activity was assessed using a NF-κB-dependent luciferase assay (Figure 8). Towards this end, A375 and LOX cells were incubated for 8 or 24 hours with the IKKα inhibitor, BAY32-5915, or with the previously described IKKβ inhibitor, KINK-1, as a comparator (Schön et al. 2008). Two hours prior to the luciferase assay, NF-κB was induced by TNFα treatment. In these experiments, KINK-1, dramatically diminished constitutive and TNFα stimulated NF-κB activity. Constitutive NF-κB activity was inhibited to values below the detection threshold ($P<0.001$ compared to vehicle-treated controls). Likewise, TNFα-induced activity was decreased by 96 % ($P<0.001$ compared to vehicle-treated cells). In contrast, no alteration of NF-κB was detected when the IKKα inhibitor, BAY32-5915, was used in luciferase assays (Figure 8).

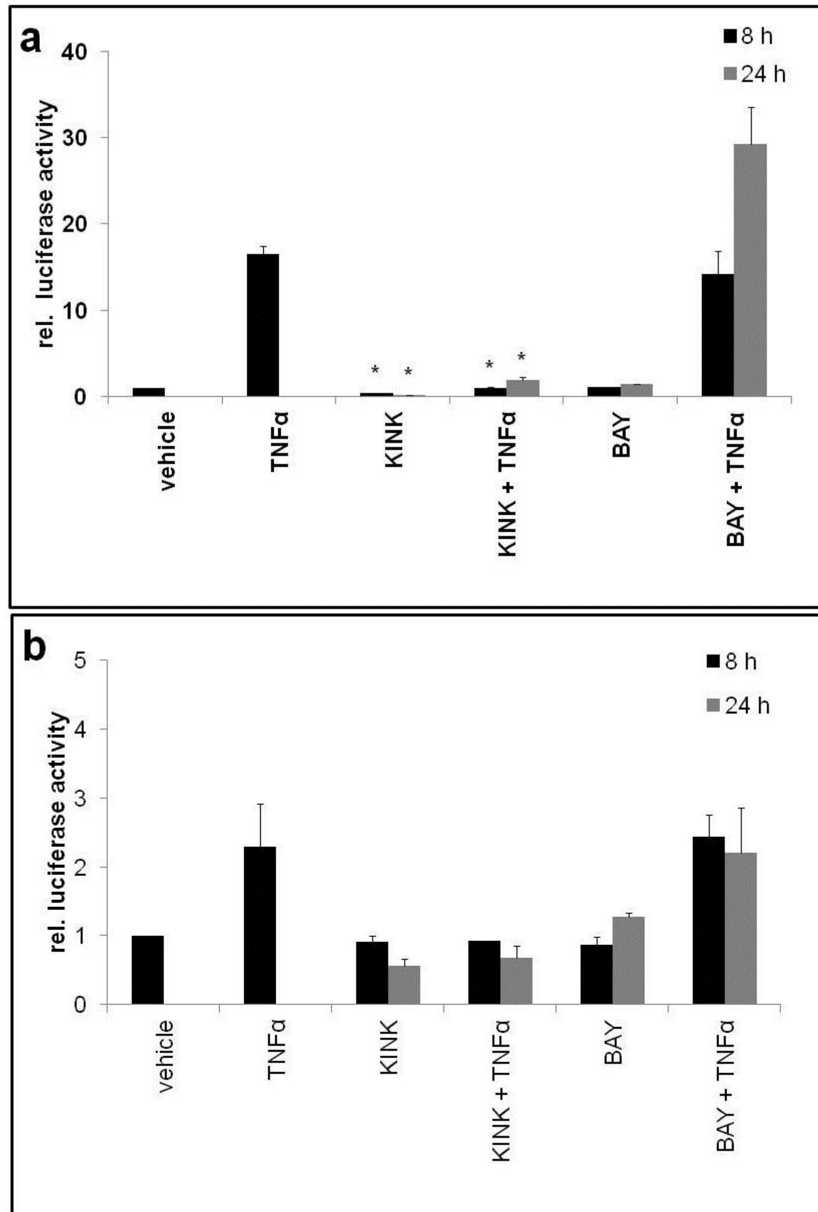


Figure 8: The IKKα inhibitor, BAY32-5915, affects neither constitutive nor TNFα-induced NF-κB activity in melanoma cells.

A373 (a) and LOX (b) melanoma cells, respectively, were transfected with luciferase specific plasmids. On the next day, the cells were incubated with 10 µM KINK-1 (KINK) or with 200 µM BAY32-5915 (BAY) for 8 (black bars) or for 24 (gray bars) hours. NF-κB was stimulated with 20 ng/ml rhTNFα for two hours prior to the luciferase assay. The NF-κB dependent luciferase activity was normalized to vehicle treated cells. There is only one bar for vehicle treated and for TNFα stimulated cells because all results were normalized to vehicle-treated cells. The values display the average of three independent experiments, each performed in triplicate (±SEM). * = p<0.005 compared to treatment without KINK-1.

In the next series of experiments, it was assessed whether treatment of melanoma cells with KINK-1 or BAY32-5915 influenced doxorubicin-induced NF-κB activation. Toward this end, vehicle or doxorubicin-treated (1 μM) melanoma cells were incubated with the IKKβ inhibitor, KINK-1, or the IKKα inhibitor, BAY32-5915, at varying concentrations for 8 hours. As shown above, treatment with doxorubicin led to clear induction of NF-κB activity. This activation was reduced by KINK-1 to a level seen in vehicle-treated cells at a concentration of 1 μM, and even below the level of constitutive activity when KINK-1 was used at concentrations of 5 μM or 10 μM, respectively (Figure 9). In contrast, BAY32-5915 did not block doxorubicin-induced or constitutive NF-κB activity, even at concentrations as high as 200 μM (Figure 9).

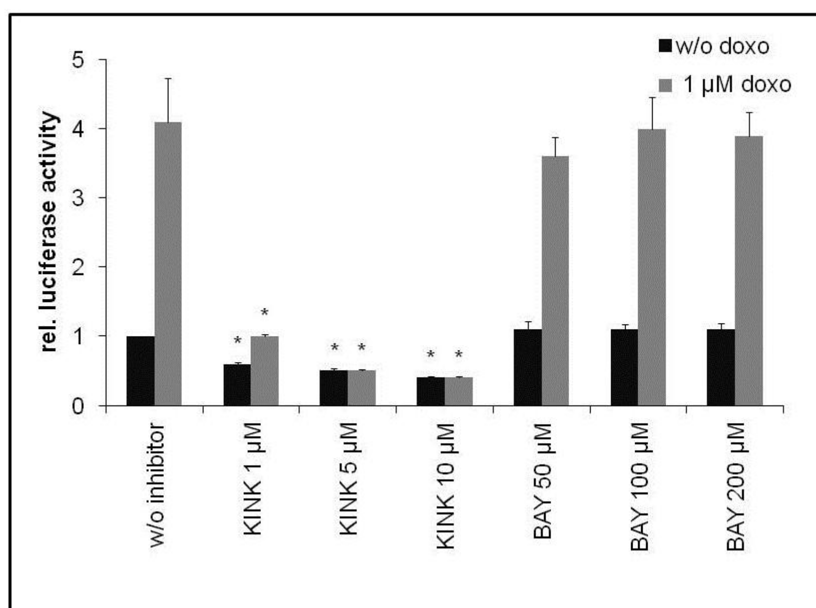


Figure 9: IKKβ inhibition by KINK-1, but not IKKα inhibition by BAY32-5915, reduced doxorubicin-induced NF-κB activation.

The NF-κB activation by doxorubicin (doxo) in combination with KINK-1 (KINK) and BAY32-5915 (BAY) was examined at the indicated concentrations using a specific NF-κB-dependent luciferase assay in the human melanoma line A375 (black bars: without doxorubicin; gray bars: with 1 μM doxorubicin). The melanoma cells were cultured for 8 hours in the presence of the drugs. KINK-1, but not BAY32-5915, inhibited doxorubicin-induced NF-κB activity in a dose-dependent manner. The values show the average of three independent experiments, each performed in triplicate (±SEM). * = $p<0.01$ compared to treatment with doxorubicin alone.

No effect on NF-κB activity was detected when human melanoma cells were treated with cisplatin and the IKK inhibitors. Again, only KINK-1, but not BAY32-5915, led to decrease of the NF-κB activity in a dose dependent manner (Figure 10).

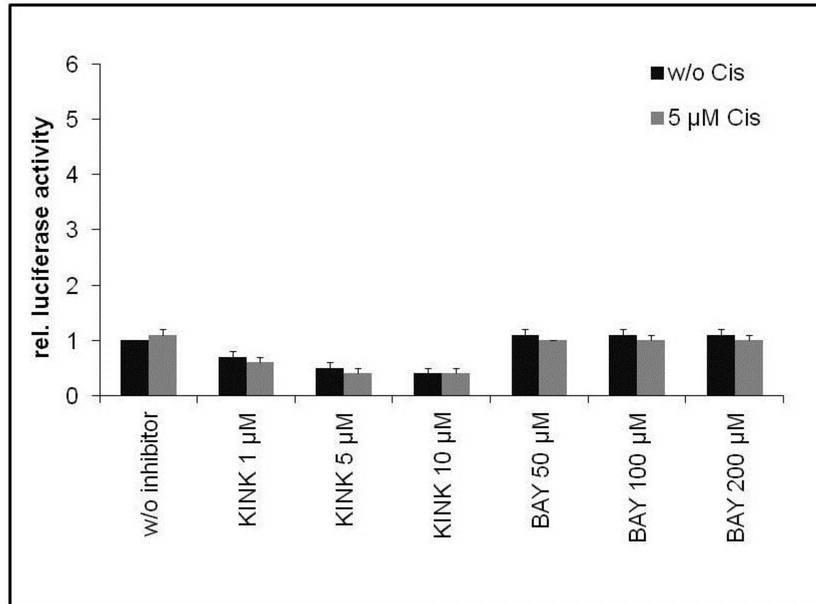


Figure 10: The combination of cisplatin with either of the IKK inhibitors did not affect NF-κB activity compared to treatment with cisplatin alone.

A375 cells were treated with (gray bars) or without 5 µM cisplatin (black bars) in combination with the IKKβ inhibitor KINK-1 (KINK) or the IKKα inhibitor BAY32-5915 (BAY) at the indicated concentrations. The values show the average of three independent experiments, each performed in triplicate (±SEM).

Based upon the induction of several tumor-promoting gene products in melanoma cells by doxorubicin (Figure 6), it was assessed whether KINK-1 or BAY32-5915 affected the doxorubicin-increased transcription of these genes. Again, melanoma cells were incubated with 1 µM doxorubicin in combination with the IKKβ or the IKKα inhibitor, respectively, at different concentrations (Figure 11). It was found that treatment with the IKKβ or IKKα inhibitors (KINK-1 or BAY32-5915) alone did not affect the transcription of the genes studied. As expected, treatment with doxorubicin stimulated the transcription of *IL-6*, *CXCL8*, *CXCL1* and *CCL2* in A375 cells as detected by RT-PCR. Interestingly,

combination with KINK-1 completely abrogated this transcriptional increase of *IL-6*, *CXCL8* and *CCL2*, while the transcription of *CXCL1* was reduced to a level even below the constitutive transcription. In contrast, no effect on gene transcription was seen when doxorubicin was combined with the IKKα inhibitor, BAY32-5915 (Figure 11).

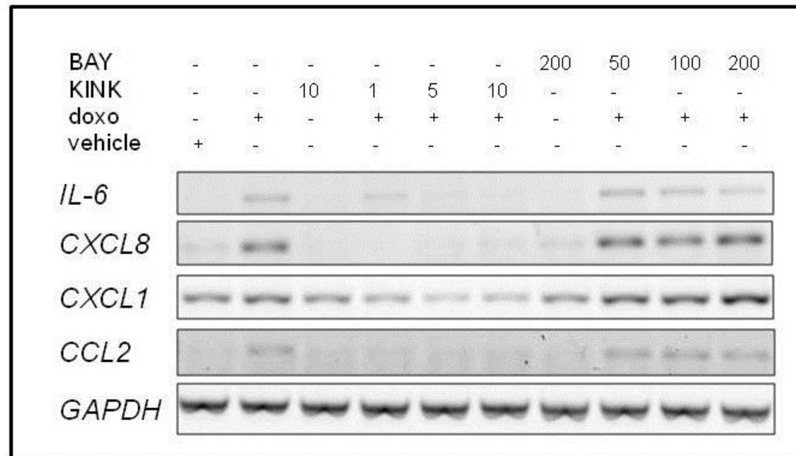


Figure 11: The IKKα inhibitor, BAY32-5915, did not reduce doxorubicin-induced transcription of NF-κB-regulated genes.

The response to incubation with 1 µM doxorubicin in combination with KINK-1 or BAY32-5915 at the indicated concentrations (µM) for 4 hours was examined in human melanoma cell line A375 by semi-quantitative RT-PCR. The combination with KINK-1 reduced the doxorubicin-induced transcription of the NF-κB regulated genes *IL-6*, *CXCL8*, *CXCL1* and *CCL2*. This experiment was repeated twice with similar results.

On the cellular level, activation of NF-κB has an anti-apoptotic effect mediated by a plethora of NF-κB-regulated gene products including the ones up-regulated by doxorubicin (Karin 2006). Previous studies have shown that inhibition of NF-κB increases the susceptibility of cancer cells to chemotherapeutic treatment (Schön et al. 2008; Bednarski et al. 2009; Amschler et al. 2010). Thus, induction of apoptosis by doxorubicin in combination with KINK-1 or BAY32-5915 was assessed by measuring the generation of histone-bound DNA fragments. A375 melanoma cells were exposed to doxorubicin alone or in combination with the IKKβ inhibitor, KINK-1, or the IKKα inhibitor, BAY32-5915 (Figure 12). Indeed, doxorubicin induced

apoptosis was significantly increased when IKKβ was blocked by KINK-1. The apoptosis rate increased 1.7-fold at a KINK-1 concentration of 5 µM ($p<0.02$) and 2-fold at 10 µM KINK-1 ($p<0.02$) compared to cells treated with doxorubicin alone. In contrast, combination of doxorubicin and BAY32-5915 did not lead to increased apoptosis of melanoma cells, even at concentrations of BAY32-5915 as high as 200 µM (Figure 12).

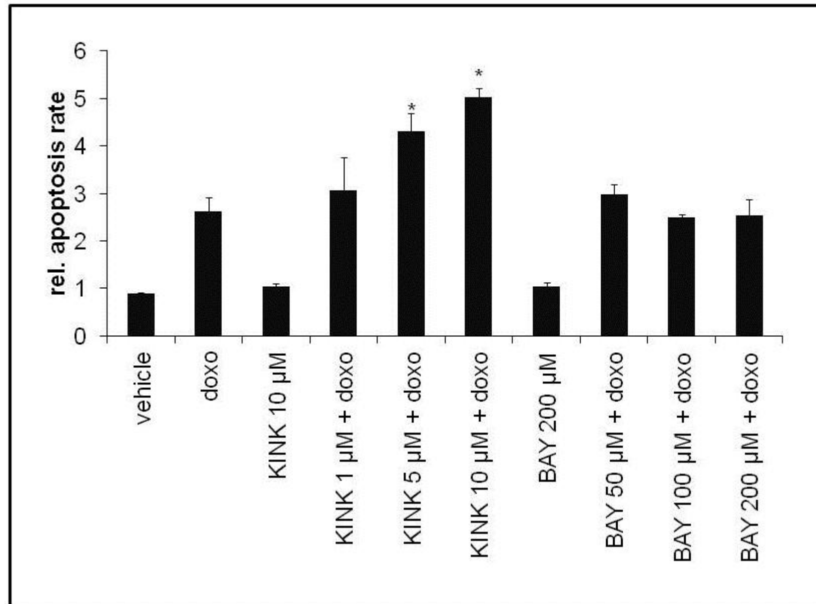


Figure 12: Doxorubicin-induced apoptosis was increased significantly when the cytostatic was combined with the IKKβ inhibitor, KINK-1, but not with the IKKα inhibitor, BAY32-5915.

A375 melanoma cells were incubated for 24 hours with doxorubicin (0.5 µM) alone or in combination with KINK-1 (KINK) or BAY32-5915 (BAY) at the indicated concentrations. Apoptosis was assessed by ELISA measuring the generation of histone-bound DNA fragments, and the measured values were normalized to vehicle-treated cells. Apoptosis of melanoma cells induced by doxorubicin was increased when the IKKβ inhibitor, KINK-1, but not the IKKα inhibitor, BAY32-5915, was present. The values show the average of three independent experiments, each performed in triplicate (±SEM). * = $p<0.02$ compared to treatment with doxorubicin alone.

Prompted by a previous report that the combination of cisplatin and the NF-κB inhibitor, BAY11-7085, which dephosphorylates IkBα, increased apoptosis of human ovarian cancer cells (Mabuchi et al. 2004), the effect of cisplatin induced apoptosis rate in combination with the IKKβ inhibitor, KINK-1,

or the IKKα inhibitor, BAY32-5915, were investigated. However, the combination of 5 μM cisplatin with varying concentrations of KINK-1 or BAY32-5915 did not affect the cisplatin induced apoptosis (Figure 13).

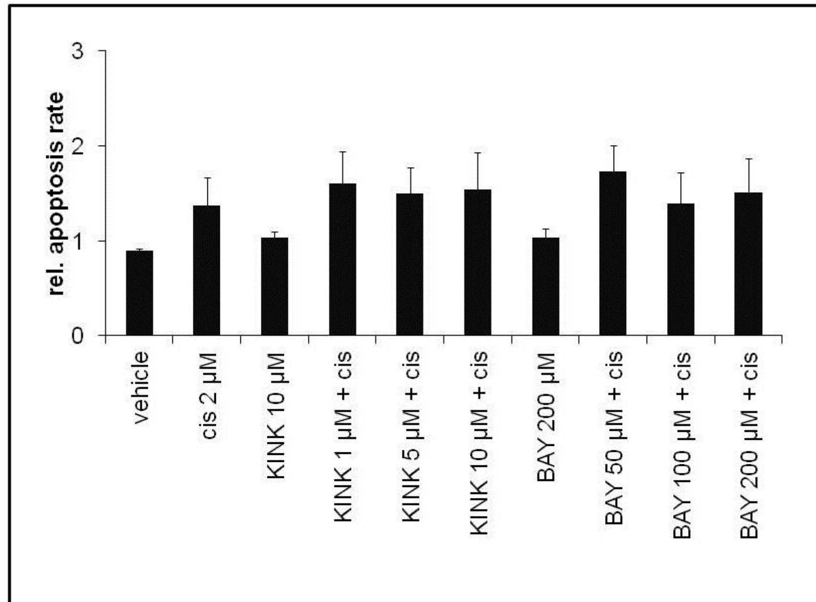


Figure 13: Cisplatin-induced apoptosis was increased neither in combination with KINK-1 nor with BAY32-5915.

A375 melanoma cells were incubated for 24 hours with 5 μM cisplatin or a combination of cisplatin with KINK-1 (KINK) or with BAY32-5915 (BAY) at the indicated concentrations. Apoptosis was assessed by ELISA measuring the generation of histone-bound DNA fragments, and the measured values were normalized to vehicle treated cells. Apoptosis of melanoma cells induced by cisplatin was not increased in combination with the IKKβ inhibitor, KINK-1, or the IKKα inhibitor, BAY32-5915, respectively. The values show the average of three independent experiments, each performed in triplicate (±SEM).

The capacity of melanoma cells to metastasize necessitates migratory behavior and has been shown before that IKKα depletion is sufficient to inhibit cell migration in prostate cancer cells (Mahato et al. 2010). Based on this notion, the effect of the IKKα inhibitor, BAY32-5915, on migration of human melanoma cells was assessed in scratch assays. The assays were performed in the presence or absence of BAY32-5915 for 72 hours, and the cultures were monitored microscopically (Figure 14). Of note, the ability of A375 or LOX

melanoma cells to migrate was not affected by treatment with the IKKα inhibitor (Figure 14).

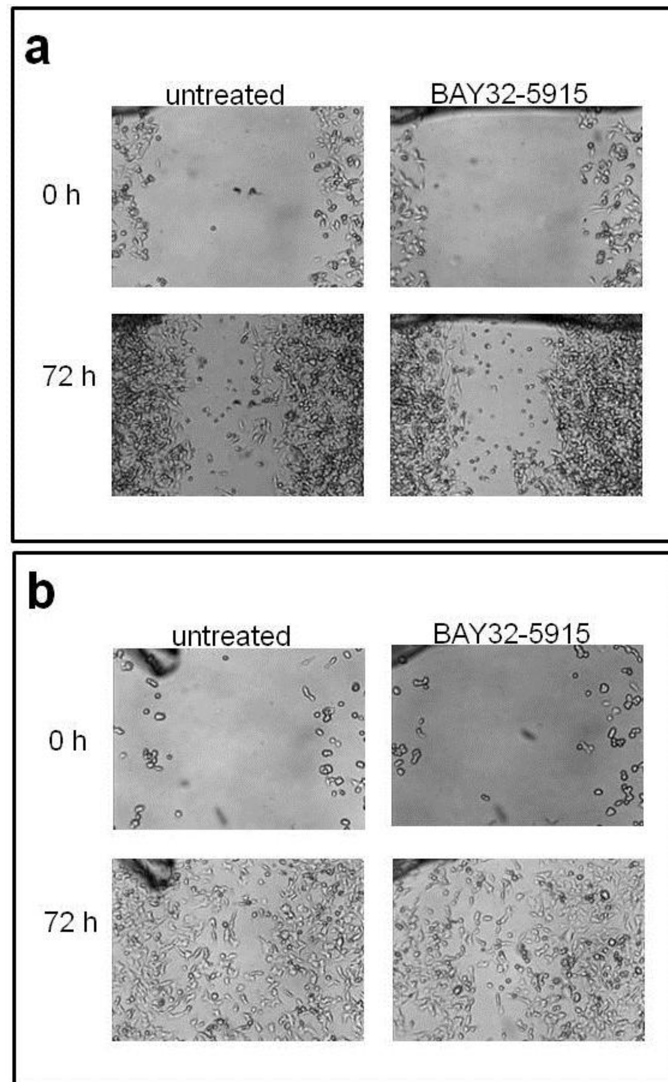


Figure 14: Migration of melanoma cells was not affected in the presence of the IKKα inhibitor, BAY32-5915.

A375 (a) and LOX (b) melanoma cells were seeded in culture inserts with defined gaps between the wells. The inserts were removed once the cells were attached, and the migration into cell free gap was documented for 72 hours. The migration of vehicle-treated cells was not distinguishable from that of BAY32-5915-treated cells. The pictures are representative for three independent experiments. Similar results were obtained with LOX melanoma cells.

In summary, the IKKβ inhibitor, KINK-1, inhibited constitutive, TNFα- and doxorubicin-induced NF-κB activity, abrogated doxorubicin-induced expression of the NF-κB-dependent gene products, IL-6, CXCL8, CXCL1 and CCL2, and

increased the apoptotic susceptibility of human melanoma cells to doxorubicin. In contrast, the IKKα inhibitor, BAY32-5915, did not inhibit NF-κB activity nor any of the NF-κB-associated functions tested. The lacking effect of the small-molecule IKKα inhibitor, BAY32-5915, suggested at least two ways of explanation, which are not mutually exclusive: First, it was possible that the compound was not active in melanoma cells, even though a profound and specific effect could be demonstrated in cell-free experimental systems (Pletz et al. 2012). Second, it was possible that IKKα did not play a decisive role for NF-κB activity and subsequent functions in melanoma cells. Both hypotheses were addressed in the subsequent experiments.

7.4 The IKKα regulated processing of p100 to p52 in B cells is not blocked by the IKKα inhibitor, BAY32-5915

It is known that the activity of the alternative NF-κB pathway is necessary for B cell maturation (Kaisho et al. 2001; Dejardin et al. 2002). In this pathway, the activation of the NF-κB inducing kinase (NIK) by CD40L, BAFF or lymphotoxin-β induces IKKα phosphorylation, and this results in processing of the NF-κB precursor, p100, to p52 (Senftleben et al. 2001; Dejardin et al. 2002; Bonizzi et al. 2004). To address the question whether BAY32-5915 was inactive in intact cells, the above pathway was employed as a positive control model system.

A modified HeLa cell line, HeLa-mCD40L-hBAFF, expresses murine CD40 ligand and human BAFF on the cell surface and also secretes these factors into the supernatant. The human B lymphoma cell line SU-DHL-4 was co-cultured with HeLa-mCD40L-hBAFF or treated with the supernatant of HeLa-mCD40L-hBAFF culture for different periods of time. The processing of p100 to p52 was assessed by Western blot (Figure 15). A shift from p100 to p52 was clearly detected after 4 hours of co-culture of the B lymphoma cells with HeLa-mCD40L-hBAFF (Figure 15); while in SU-DHL-4 cells treatment with

supernatant of HeLa-mCD40L-hBAFF started the processing later and weaker (Figure 15).

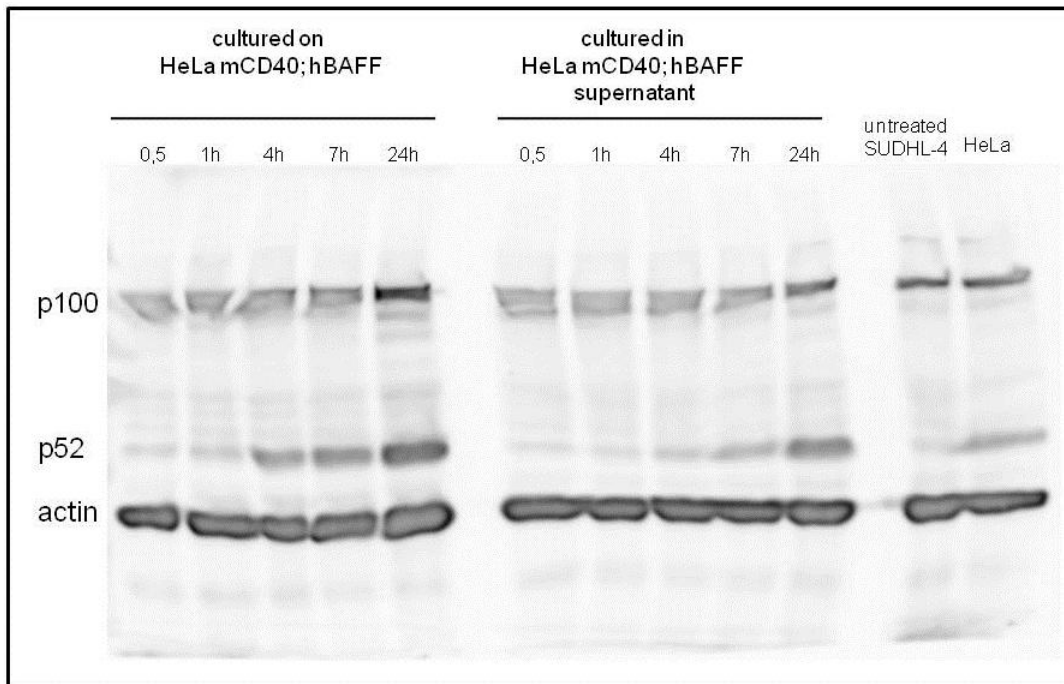


Figure 15: The alternative NF-κB pathway is activated in B lymphoma cells by co-culturing with modified HeLa cells stably expressing murine CD40 ligand and human BAFF.

The B lymphoma cell line, SU-DHL-4, was co-cultured with the adherent human cancer cell line HeLa, which was modified to stably express murine CD40 ligand and human BAFF. In parallel, SU-DHL-4 cells were treated with supernatant of the HeLa-mCD40L-hBAFF. The whole cell lysates of the suspension cells were examined by Western blot for the processing of p100 to p52. The processing of p100 was induced after 4 hours of co-culture or after 7 hours of exposure to the conditioned supernatant. The p100/p52 status of untreated SU-DHL-4 and HeLa-mCD40L-hBAFF was also examined. The Western blot is representative for two independent experiments showing similar results.

To test the efficiency of the IKKα inhibitor, BAY32-5915, the SU-DHL-4 cells were co-cultured with HeLa-mCD40L-hBAFF in the presence of the inhibitor for 4 hours. A block of the processing of p100 by BAY32-5915 was expected, but this occurred only at very high concentrations of BAY32-5915 of ≥ 1000 µM (Figure 16).

Figure 16: The IKKα inhibitor, BAY32-5915, impairs processing of p100 to p52 in co-culture of B lymphoma with HeLa-mCD40L-hBAFF only at high concentrations.

SU-DHL-4 cells were co-cultured on HeLa-mCD40L-hBAFF for 4 hours in presence of BAY32-5915 at the indicated concentrations. Western blot analysis revealed moderate inhibition of the IKKα-dependent processing of p100 to p52 by BAY32-5915 treatment only at very high concentrations. The experiment shown is representative for two independent experiments with similar results.

The weak, if any, inhibition of p100 processing by BAY32-5915 gave rise to further experiments addressing the function of IKKα in melanoma cells by means of IKKα-directed siRNA experiments.

7.5 Reduction of IKKα protein expression has a moderate impact on NF-κB activity

Previous studies have suggested a role for IKKα in prostate cancer cells, where IKKα controls the invasiveness by repressing the tumor suppressor maspin (Luo et al. 2007; Mahato et al. 2010). Moreover, IKKα has a critical role for chemoresistance against doxorubicin in human fibroblasts (Bednarski et al. 2008). Further it was shown that IKKα promotes cell growth in human cancer cell lines by switching the binding preference of the transcription co-factor CREB binding protein (CBP) from p53 to the NF-κB factor p65 (Huang et al. 2007). IKKα regulates the mitotic phase of the cell cycle in HeLa cells by

affecting Aurora A phosphorylation (Prajapati et al. 2006). The high intrinsic activity of IKKβ kinase may also be inhibited by IKKα (O'Mahony et al. 2000). Recently, a novel function of IKKα in the control of cyclin D1 synthesis and degradation was described (Song et al. 2010; Kwak et al. 2011).

To further investigation a potential role of IKKα in melanoma cells, it was silenced in A375 and LOX melanoma cells by transfection with specific siRNA constructs (Figure 17). As demonstrated by densitometric analysis of the Western blots, IKKα protein expression was reduced by approximately 50 % in both cell lines.

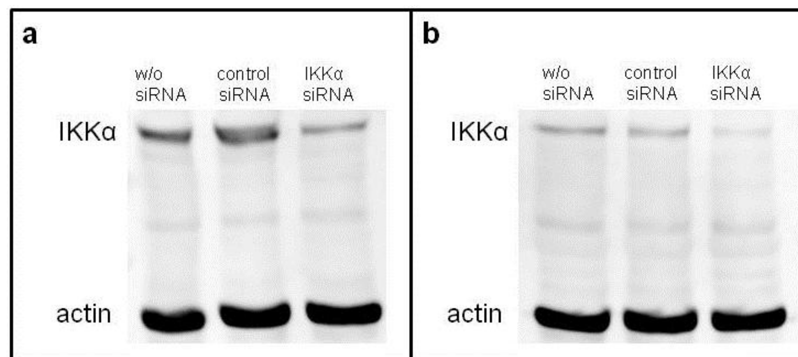


Figure 17: Silencing of IKKα by siRNA reduced IKKα expression by approximately 50 %.

The human melanoma cell lines were transfected with the indicated siRNA constructs (a: A375 with 25 nM and b: LOX cells with 50 nM siRNA) and cultured for 48 hours. Expression of IKKα and, as an internal control, actin was assessed by Western blot.

To analyze the impact of IKKα on doxorubicin-induced NF-κB activation, melanoma cells were co-transfected with siRNA directed against IKKα and the vectors required for the NF-κB-dependent luciferase assay for 48 hours. The subsequent exposure of melanoma cells (A375 and LOX) to doxorubicin induced NF-κB activity in cells without siRNA, with control siRNA and with IKKα siRNA treatment; however, the doxorubicin-induced NF-κB activation was reduced by approximately 40 % in melanoma cells with reduced IKKα synthesis as compared to the controls (Figure 18). Additionally, silencing of IKKα down

regulated constitutive NF-κB activity by approximately 50 % in A375 melanoma cells, but not in LOX cells. Finally, IKKα downregulation did not appear to have an impact on TNFα stimulated NF-κB activity (Figure 18).

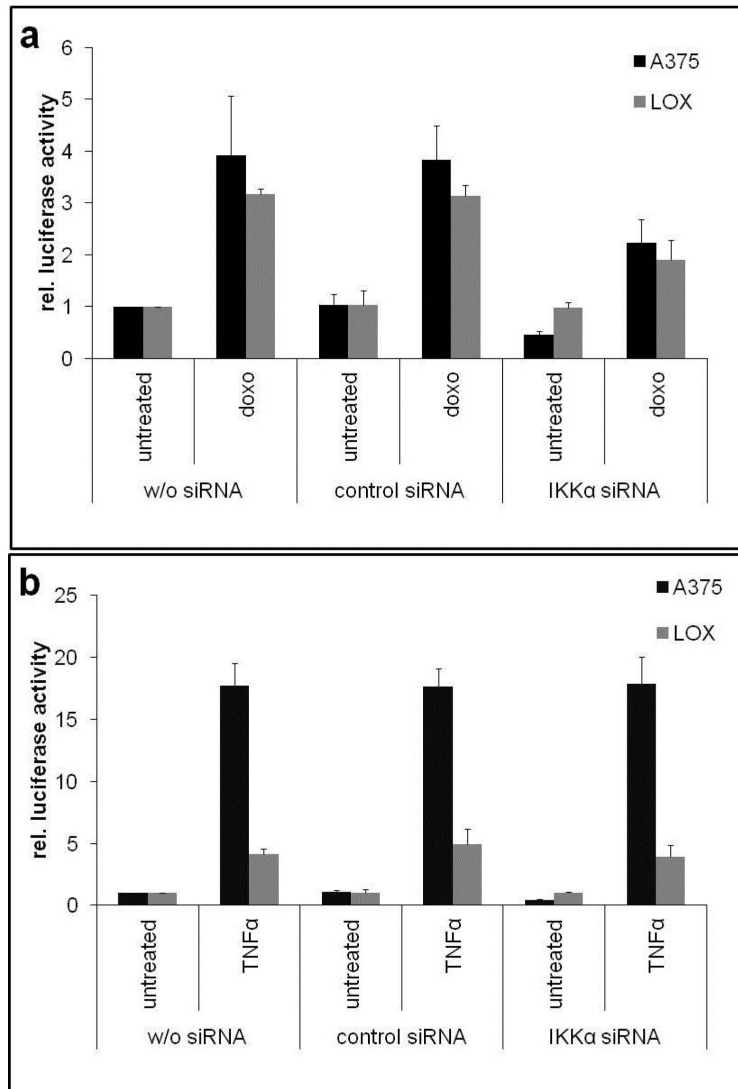


Figure 18: Silencing of IKKα has a limited effect on doxorubicin-induced, but not on TNFα-stimulated, NF-κB activation.

A375 and LOX melanoma cells were co-transfected with siRNA constructs and with vectors required for the NF-κB-dependent luciferase assay for 48 hours. Cells were cultured for 8 hours in the presence of 1 μM doxorubicin (a) and 2 hours with 20 ng/ml rhTNFα (b). Subsequently, NF-κB activation was determined by NF-κB-dependent luciferase assay. The constitutive NF-κB activity was reduced by IKKα-directed siRNA by approximately 50 % in A375 cells, but not in LOX cells. Additionally, silencing of IKKα resulted in a down-regulation of doxorubicin-induced NF-κB activation in both melanoma cell lines. No impact on TNFα stimulated NF-κB activation was measured. The values show the average of three independent experiments (±SEM).

To compare whether reduction of kinase synthesis had a similar effect as kinase inhibition by small molecules, IKKβ, too, was diminished by siRNA. The reduction of IKKβ level by siRNA was more difficult to establish as compared to IKKα; it could only be achieved when a second siRNA treatment was performed after 48 hours for another 24 hours. In A375 melanoma cells, this boosted treatment reduced the cellular IKKβ level by approximately 75 % (Figure 19).

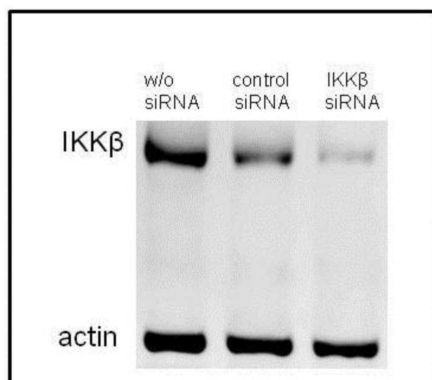


Figure 19: Dual transfection with IKKβ–directed siRNA reduced IKKβ expression by approximately 75 %.

The human melanoma cell line A375 was transfected with 100 nM of the indicated siRNA constructs and cultured for 48 hours, then the siRNA treatment was repeated and the cells were cultured for another 24 hours. The expression of IKKβ and actin was assessed by Western blot.

The next series of experiments was performed to assess the constitutive as well as the induced NF-κB activity in melanoma cells, whose IKKβ activity reduced by double transfection of siRNA. Towards this end, A375 melanoma cells were treated with siRNA twice followed by 8 hours of doxorubicin treatment or 2 hours of exposure to TNFα. It was found that IKKβ inhibition by siRNA significantly reduced the endogenous NF-κB activity by 67 % and the doxorubicin-induced NF-κB activity by 70 % ($P < 0.01$ in both cases), while the TNFα-stimulated NF-κB activity was not significantly reduced by only approximately 30 % as compared to the respective controls (Figure 20). These data suggested that the reduction of IKKβ by siRNA had similar, albeit somewhat weaker, effects on NF-κB activity as treatment with the small-

molecular inhibitor, KINK-1, a finding that might be explained by the residual IKKβ activity in siRNA-treated cells.

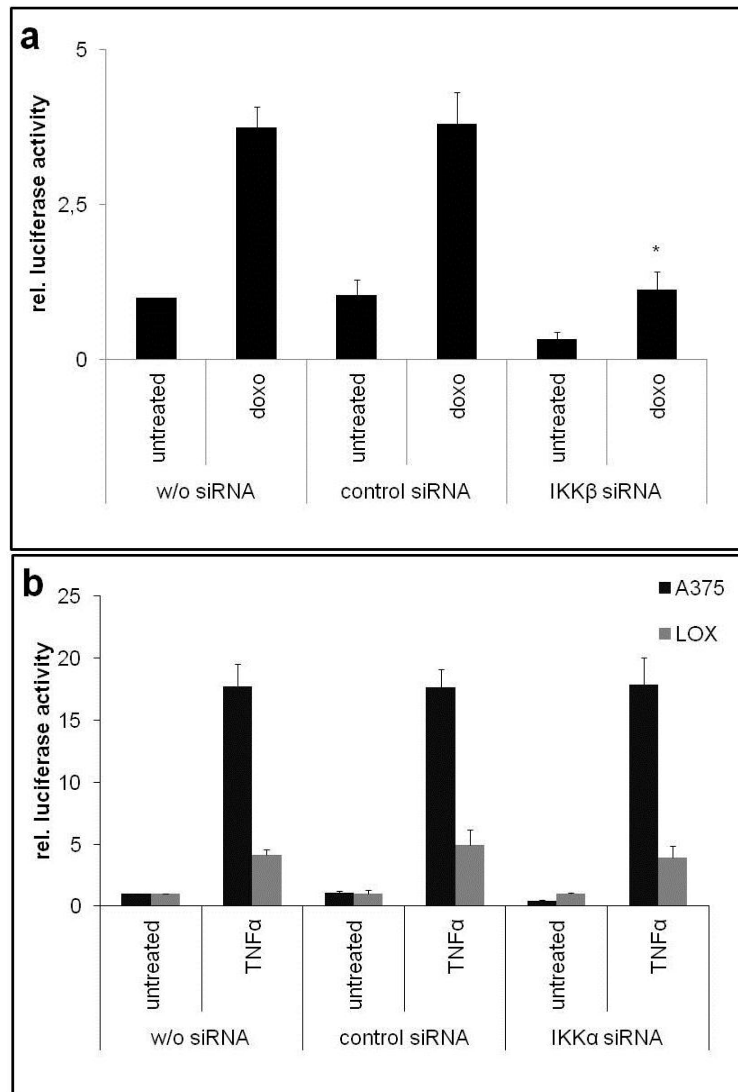


Figure 20: Silencing of IKKβ reduces constitutive, TNFα- and doxorubicin-induced NF-κB activation.

A375 melanoma cells were transfected with siRNA constructs for 48 hours followed by an additional treatment with siRNA constructs and the vectors of the NF-κB-dependent luciferase assay for 24 hours. Cells were cultured for 8 hours in the presence of 1 µM doxorubicin (a) or 2 hours with 20 ng/ml rhTNFα (b). Subsequently, NF-κB activation was determined by NF-κB-dependent luciferase assay. The constitutive NF-κB activity was reduced by IKKβ-directed siRNA by 67 %, the doxorubicin induced activity by 70 % and the TNFα stimulated activity only by approximately 30 %. The values show the average of three independent experiments (±SEM). * = $p<0.01$ compared to control siRNA treated cells.

7.6 Constitutive processing of p100/p52 in some melanoma cells

Given that IKKα is thought to be particularly involved in the processing of the NF-κB precursor p100 to p52, this proteolytic process was examined as an indirect parameter for the activation status of IKKα in melanoma cells. When whole cell lysates of 8 unstimulated melanoma lines were analyzed by Western blot (Figure 21), exclusively LOX cells revealed a clear shift from p100 to p52, while Brown cells showed a weaker, if any, signal and the other lines were virtually negative (Figure 21).

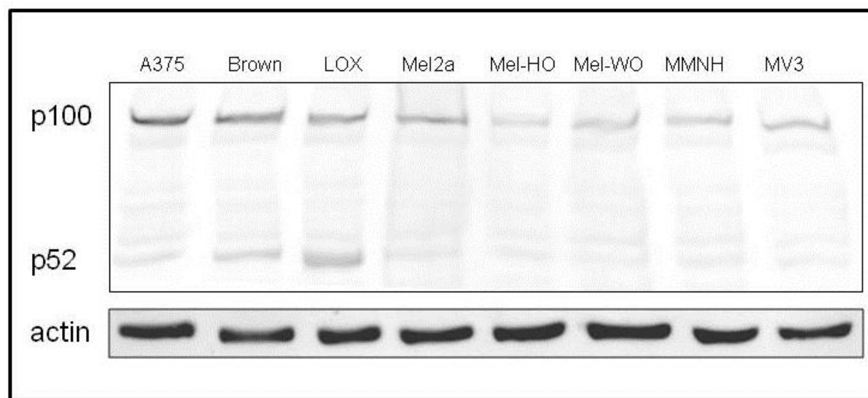


Figure 21: Constitutive processing of p100 to p52 was found in the human melanoma cell line LOX.

Whole cell lysates of the melanoma cell lines A375, Brown, LOX, Mel2a, Mel-Ho, Mel-Wo, MMNH and MV3 were analyzed by Western blot for p100/p52. Expression of p100 was detected in all cell lines, while a clear p52 signal was seen only in LOX and weakly in Brown cells. Actin was detected as loading control. The Western blot shown is representative for two independent experiments showing similar results.

The processing of p100 leads to generation and, consecutively, nuclear translocation of p52, the latter in form of the RelB/p52 heterodimer (Senftleben et al. 2001; Solan et al. 2002). Thus, the distribution of p100/p52 was assessed in all eight melanoma cell lines by immunofluorescence staining of p100/p52. In good confirmation of the p100/p52 Western blot results, a cytoplasmic staining was detected in all cell lines and the nucleus was excluded. Again confirming

the results of the Western blot analyses, the sole exception were LOX cells, where p100/p52 signals were also detected in the nucleus (Figure 22).

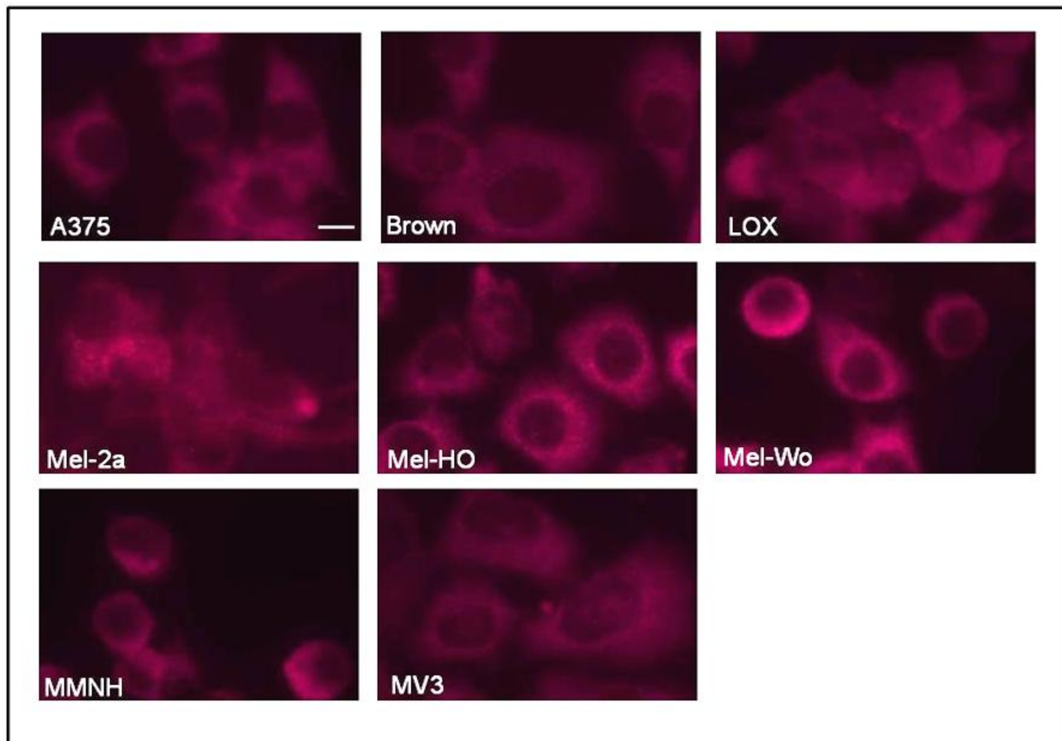


Figure 22: P100/p52 was detected in the nucleus of the LOX melanoma cell line.

Unstimulated melanoma cells were fixed and probed with anti-p100/p52 antibody followed by alexa-555 conjugated secondary antibody. P100/p52 was detected in all cell lines in the cytoplasmic and in LOX cell line even also in the nucleus. The pictures are representative for two independent experiments. Scale bars = 10 µm.

In order to confirm the differential constitutive processing and nuclear translocation of p52 in melanoma cells by a second independent method, the cytoplasmic and nuclear fractions of A375 and LOX melanoma cells were prepared and analyzed separately by Western blot. Confirming the results of the immunofluorescence staining, a clear p100 signal was identified in the cytoplasmic fraction and virtually no p52 was detected in the nuclear fraction of A375 melanoma cells (Figure 23). In contrast, both p100 and p52 were detected within the cytoplasmic fraction, and p52 was found accumulated within the

nuclear fraction of LOX cells (Figure 23). IKKα was found within the cytoplasm of both cell lines (Figure 23).

Overall, based on the processing of the NF-κB precursor p100 to p52 in unstimulated cells, the alternative NF-κB pathway appears to be constitutively active only in the melanoma cell line LOX.

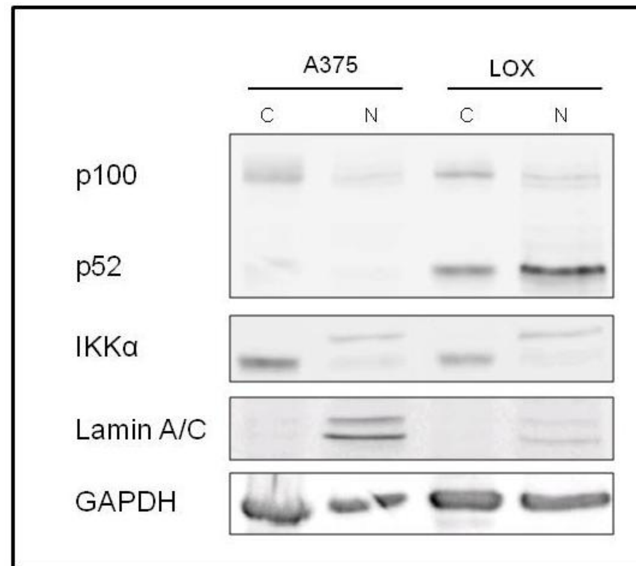


Figure 23: Constitutive processing of p100 to p52 and accumulation of p52 in the nuclear fraction of LOX melanoma cells.

The cytoplasmic (C) and nuclear (N) fractions of A375 and LOX melanoma cells were assessed for expression of p100/p52 and IKKα by Western blot. In A375 cells, p100 and IKKα were detected in the cytoplasm. In LOX cells, cytoplasmic p100 was processed to p52, the latter of which was strongly present within the nuclear fraction. IKKα was also detected in the cytoplasmic fraction of both cell lines. GAPDH was used as loading control and lamin A/C was used as a marker confirming the identity of the nuclear fraction. This Western blot is representative for two independent experiments with similar results.

7.7 Reduction of IKKα expression affects the expression of tumor progression factors

It has been shown previously that IKKα is involved in the regulation of the cell cycle through nuclear export of cyclin D1 and that it may contribute to tumor progression via repression of the tumor suppressor maspin (Albanese et al.

2003; Luo et al. 2007; Song et al. 2010). Based on that notion, the next series of experiments was designed to address the role of IKKα in the regulation of progression-associated factors in melanoma cells. In addition, it was of interest how IKKα was involved in doxorubicin-induced expression of NF-κB-regulated gene products (as depicted in Figure 6). Towards this end, melanoma cells were left untreated or were treated with control siRNA or IKKα siRNA, followed by exposure to doxorubicin or TNFα. The transcription of genes of interest was assessed by semi-quantitative RT-PCR (Figures 24).

As expected, the transcripts of the *CHUK* gene (encoding for IKKα) were clearly reduced by IKKα siRNA treatment in both A375 and LOX melanoma cells (Figure 24a and b). Surprisingly, in A375 cells the TNFα and doxorubicin-induced *IL-6*, *CCL2*, and *ICAM-1* transcription was reduced when IKKα was diminished by siRNA treatment. However, only doxorubicin- but not the TNFα-induced transcription of *CXCL8* was impaired by IKKα reduction. Further, the amount of *CCND1* transcripts, which encodes for cyclin D1, was not affected in A375 cells by NF-κB activation by TNFα and doxorubicin or IKKα-directed siRNA, respectively. The transcription of the *SERPINB5* gene (encodes for the tumor suppressor maspin) was increased by doxorubicin treatment, and the reduction of IKKα did not alter the *SERPINB5* transcription (Figure 24a).

In LOX melanoma cells, no transcription of *IL-6*, *CCL2* or *SERPINB5* genes was seen (Figure 24b). Similar to A375 cells, the transcription of *CXCL8* and *ICAM-1* was also stimulated by exposure to TNFα or doxorubicin, and IKKα reduction attenuated both, doxorubicin- and TNFα-stimulated transcription of these factors (Figure 24b). Transcription of *CCND1* was not affected by TNFα or doxorubicin, but reduced transcription was detected in IKKα siRNA transfected cells (Figure 24b). Exposure to 1 µM doxorubicin slightly reduced *CXCL1* transcription, independent of siRNA treatment.

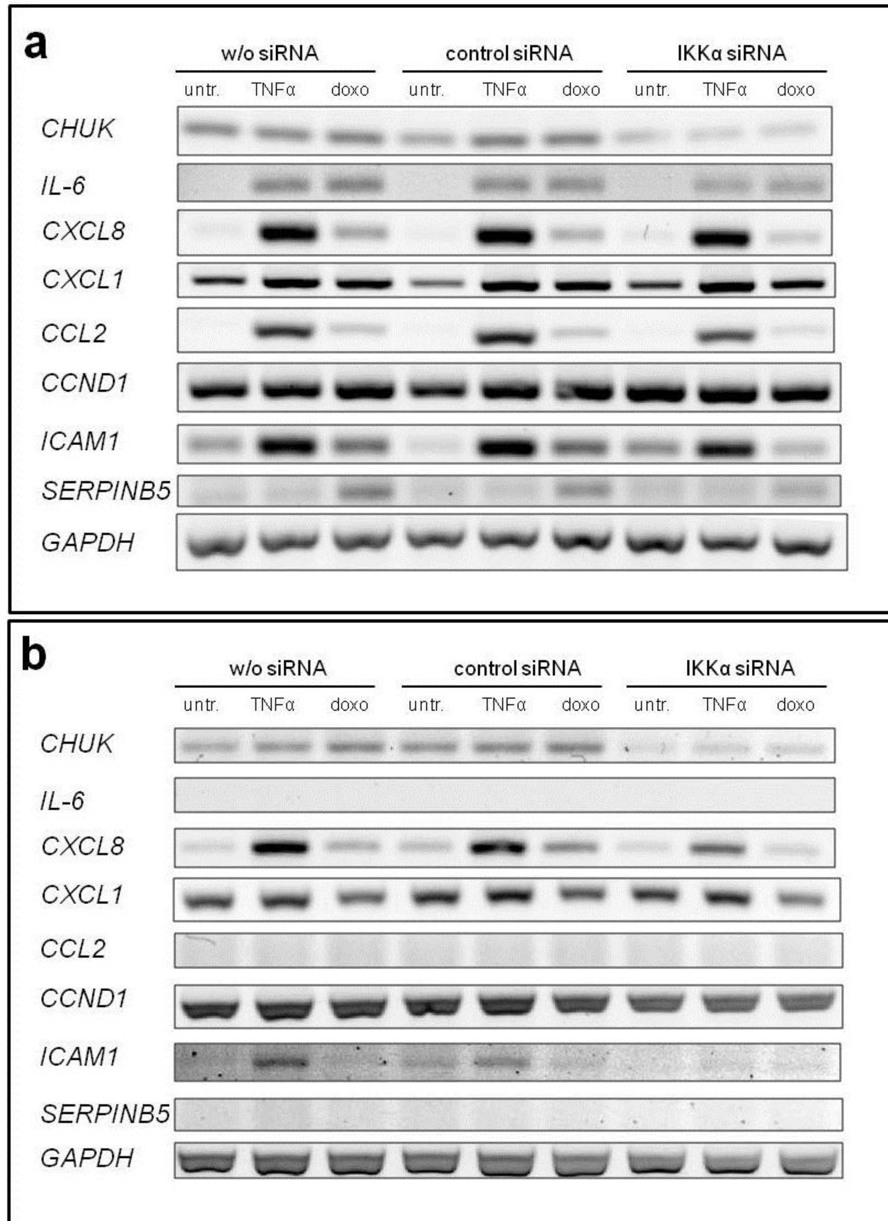


Figure 24: Reduction of IKKα transcripts resulted in a moderate inhibition of doxorubicin-and particularly TNFα-induced transcription of NF-κB-regulated genes.

Untreated melanoma cells or melanoma cells treated for 48 hours with control siRNA or IKKα-directed siRNA were exposed to 1 μM doxorubicin for 4 hours or to 20 ng/ml rhTNFα for 2 hours. The transcription of genes of interest was detected by semi-quantitative RT-PCR. The amount of IKKα transcripts was measurably reduced by IKKα-directed siRNA, but not by control siRNA. Doxorubicin-induced transcription of NF-κB-regulated genes by IKKα knockdown was reduced in accordance with the results of the NF-κB-dependent luciferase assay (Figure 18). TNFα-stimulated transcription of some genes was slightly reduced by IKKα reduction (A375: *IL-6*, *CCL2*, and *ICAM-1*; LOX: *IL-6* and *ICAM-1*).

As shown before, the reduction of IKKα expression led to a moderate decrease of doxorubicin-induced NF-κB activity and, consecutively, moderate inhibition of the transcription of some NF-κB-dependent genes (Figure 24). Notably, the TNFα-stimulated transcription of *IL-6*, *CXCL8*, *CCL2* and *ICAM-1* was reduced by IKKα depletion in both A375 and LOX cells. Moreover, in LOX cells the *CCND1* transcription was moderately decreased when IKKα was reduced. Overall, it appeared that the reduction of IKKα could confer some anti-tumoral activity through impairment of several tumor-promoting factors in melanoma cells, even though the effects on NF-κB activity were limited.

Complementing the investigations of tumor progression-related mediators, the levels of proteins involved in apoptosis, such as survivin, Bcl_{XL}, Bcl2 and Bax, were assessed. Likewise, the stabilization of cytoplasmic β-catenin was investigated because it has been shown that this may be influenced by IKKα (Senftleben et al. 2001; Albanese et al. 2003; Carayol and Wang 2006; Song et al. 2010). Again, the protein levels of untreated, control siRNA-treated or IKKα siRNA-treated melanoma cells were determined by Western blot following exposure to TNFα or doxorubicin (Figure 25). Confirming the results of previous experiments, inhibition of IKKα synthesis by IKKα siRNA treatment could be shown clearly in both melanoma cell lines (Figure 25). However, synthesis of cyclin D1, survivin, β-catenin, Bcl_{XL}, Bcl2 and Bax was not affected by siRNA treatment, by exposure to TNFα or by treatment with doxorubicin, respectively (Figure 25).

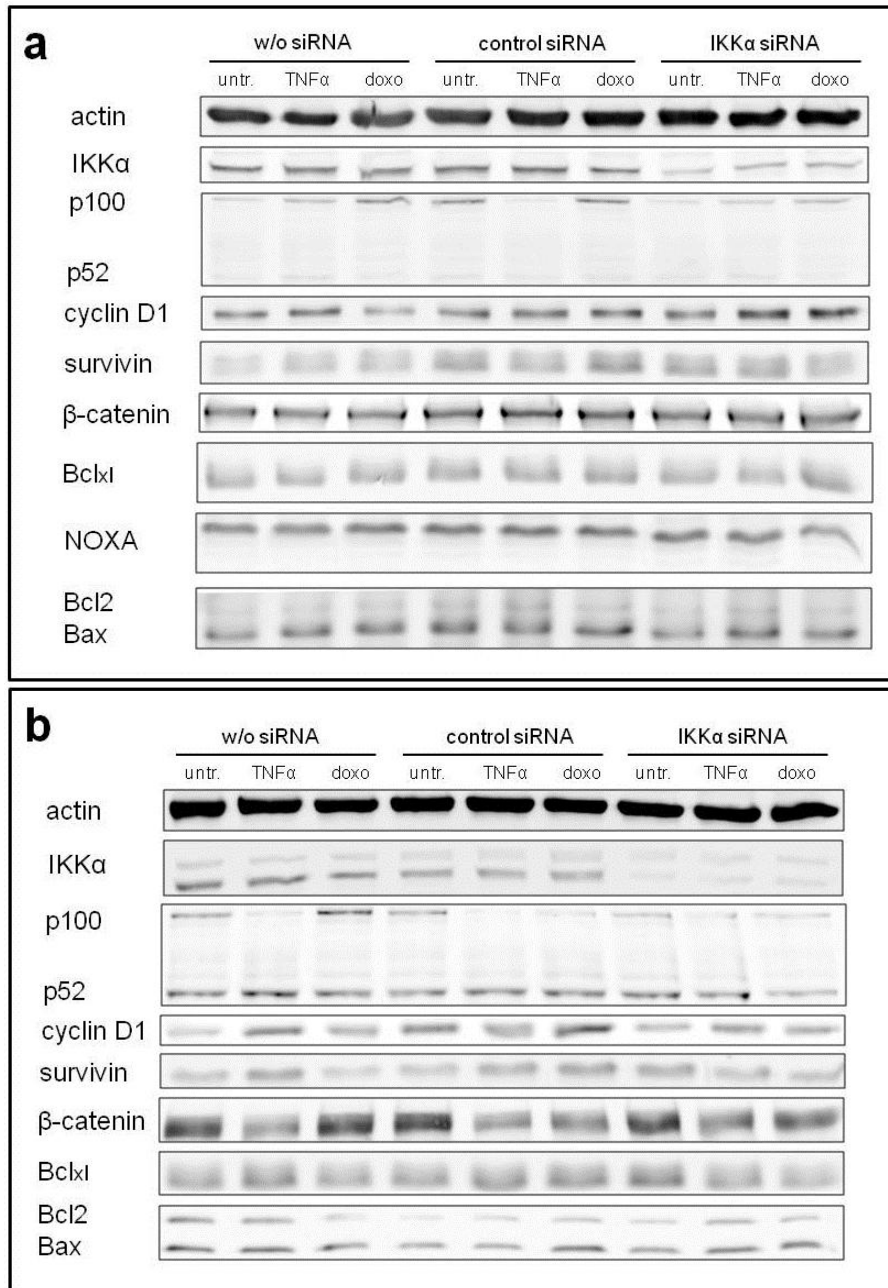


Figure 25: Reduction of IKKα synthesis did not affect synthesis of apoptosis-related gene products in human melanoma cells.

A375 (a) and LOX (b) melanoma cell lines were left untreated or were treated with control or IKKα-directed siRNA for 48 hours. Subsequently, the cells were exposed to 1 µM doxorubicin for 8 hours or to 20 ng rhTNFα/ml for 2 hours. Western blot analysis was performed using whole cell lysates. While IKKα synthesis was clearly inhibited by IKKα siRNA, no effect on any of the detected proteins could be discerned. The shown Western blots are representative for three independent experiments.

Cyclin D1 is a regulator of the G1/S-phase transition. Its overexpression or deregulation are strongly associated with tumorgenesis (Diehl 2002). Furthermore, IKKα was described to be required for the nuclear export of cyclin D1 in mouse embryonic fibroblast (Kwak et al. 2011). To address whether reduction of IKKα led to nuclear accumulation of cyclin D1 in melanoma cell lines, cyclin D1 was determined by Western blot in the cytoplasmic and nuclear fractions of IKKα siRNA-treated melanoma cells. When IKKα was reduced in A375 or LOX melanoma cells, no shift in cyclin D1 distribution was detected compared to untreated or control siRNA treated cells (Figure 26).

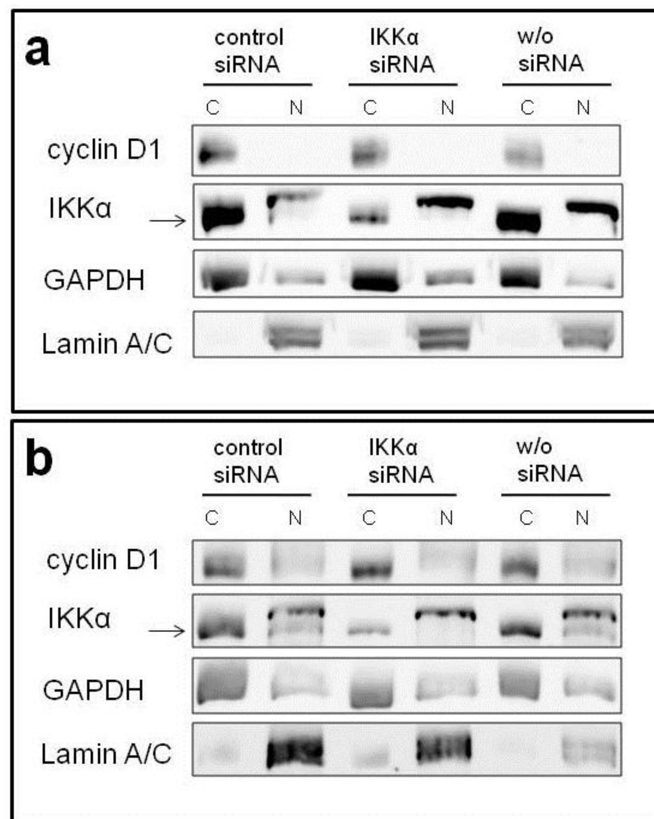


Figure 26: Reduction of IKKα by siRNA did not affect the subcellular distribution of cyclin D1 in human melanoma cells.

The cytoplasmic (C) and nuclear fractions (N) were analyzed for cyclin D1 by Western blot. In both melanoma cell lines, cyclin D1 was located in the cytoplasmic. When IKKα was reduced by siRNA treatment, no overt changes regarding cyclin D1 distribution were detected in either cell line. Lamin was detected as a control for the purity of nuclear extracts and GAPDH was determined for cytoplasmic control. The shown Western blots are representative for two independent experiments.

In vitro scratch assays examining cell migration were performed to assess the impact of IKKα reduction at the cellular level. Again, melanoma cells remained untreated or were treated with control or IKKα siRNA for 48 hours followed by scratching the cell layer and monitoring for 72 hours. Migration of IKKα siRNA-treated A375 cells was similar to that of untreated or control siRNA-treated cells (Figure 27a). In contrast, LOX cells migrated slightly slower when transfected by IKKα siRNA compared to untransfected or control siRNA-transfected cells (Figure 27b). This distinct difference was paralleled by a transcriptional down-modulation of *ICAM-1* (intercellular adhesion molecule-1, CD54; Figure 24b). However, no clear overall effect of IKKα down-modulation on cell migration could be detected.

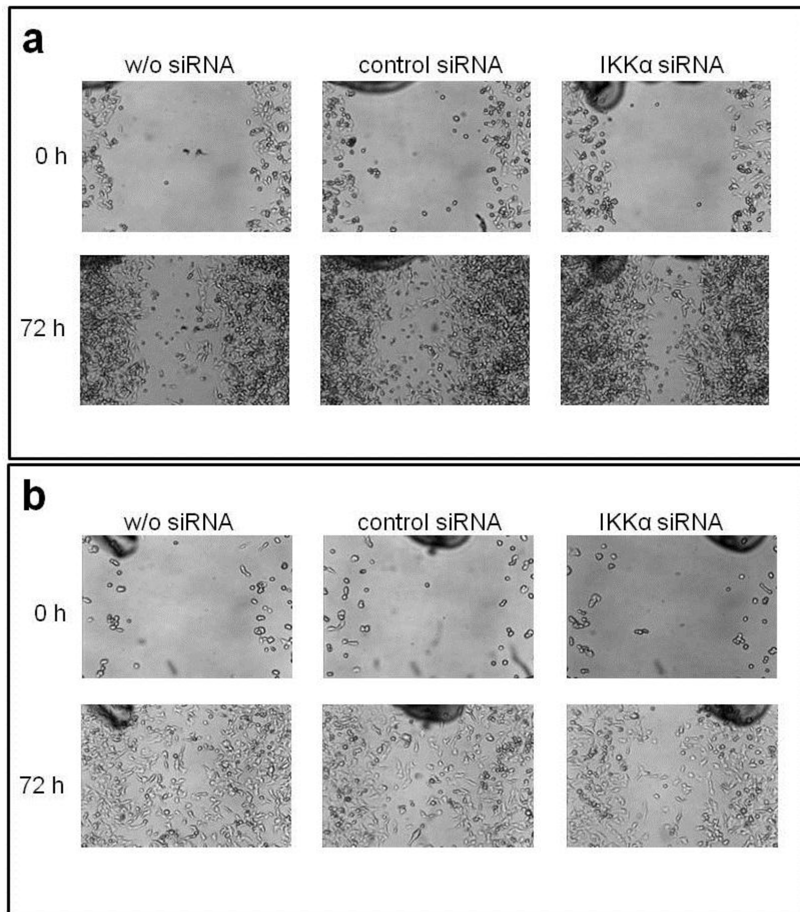


Figure 27: Migration of LOX, but not A375, melanoma cells, was reduced moderately upon treatment with IKKα-siRNA.

Melanoma cell lines A375 (a) and LOX (b) were treated with control siRNA, IKKα siRNA or remained untreated for 48 hours. Thereafter, the cell layer was scratched in a standardized fashion and migration into the cell-free area was monitored by photo documentation for 72 hours. IKKα siRNA treated LOX cells migrated slightly slower than control cells. The experiments shown is representative for three independent experiments showing similar results.

In the next series of experiments, the impact of IKKα depletion on the proliferation of melanoma cells was examined. Toward this end, melanoma cells were treated with siRNA against IKKα or control siRNA for 48 hours. Subsequently, cell proliferation was determined by cellular conversion of tetrazolium salt into a formazan product (MTT assay). The absorbance of the formazan product is proportional to the number of living cells. It was found that

neither of the two melanoma cell lines showed significant alterations of proliferation upon silencing of IKKα (Figure 28).

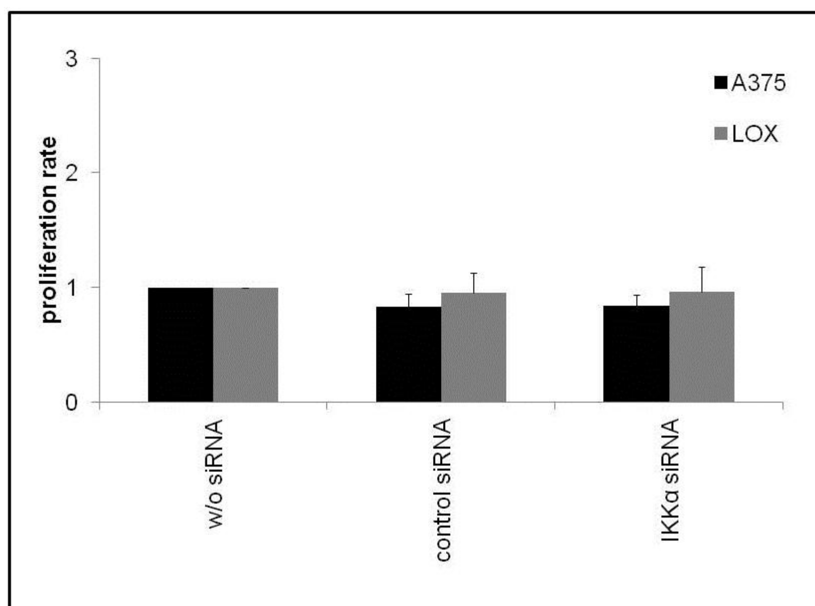


Figure 28: Reduction of IKKα by siRNA in melanoma cells did not affect cell proliferation.

The melanoma cell lines A375 (black bars) and LOX (gray bars) were transfected with IKKα or control siRNA for 48 hours. Subsequently, cell proliferation was determined using the MTT assay. The measured values were normalized to untreated cells. The values show the average of two independent experiments, each performed in triplicate (±SEM).

Based on the notion that the IKKβ inhibitor, KINK-1, increased the apoptotic response in melanoma cells when combined with doxorubicin (Figure 12), in the next series of experiments the apoptosis rate was assessed to address the question whether down-modulation of IKKα, too, would increase doxorubicin-induced apoptosis in melanoma cells. Again, melanoma cells were treated with siRNA against IKKα or control siRNA for 48 hours and, subsequently, treated with doxorubicin for 24 hours. Apoptosis was determined using an ELISA, which measures the generation of histone-bound DNA fragments. It was found that the reduction of IKKα expression did not significantly affect the apoptosis of untreated melanoma cells (Figure 29). The

exposure of melanoma cells to a suboptimal concentration (i.e., 0.5 µM, which is well below the LD_{50} concentration) of doxorubicin resulted in increases of apoptosis of 1.4-fold in A375 and 2.2-fold in LOX melanoma cells, respectively. However, the apoptotic responses of either cell line achieved by doxorubicin treatment did not change when IKKα levels were reduced by siRNA (Figure 29).

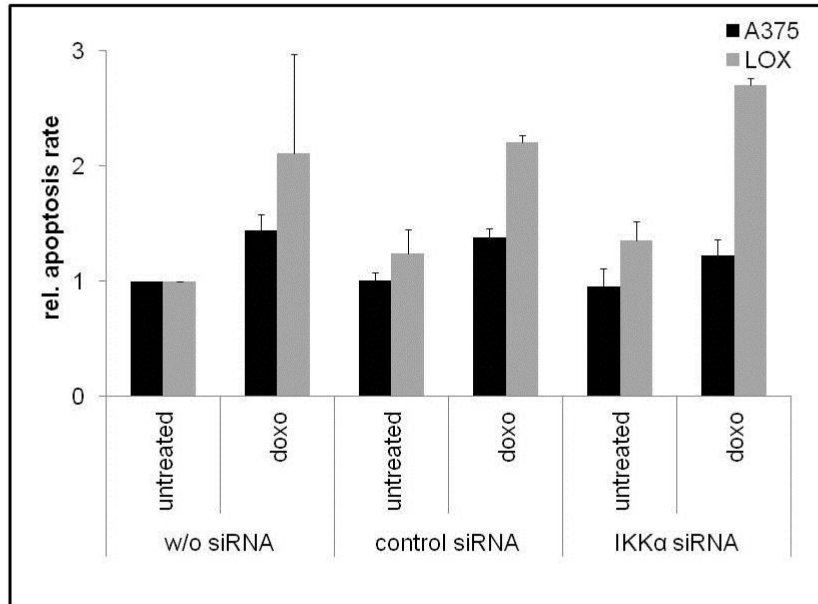


Figure 29: Reduction of IKKα in melanoma cells did not increase the doxorubicin-induced apoptosis.

A375 (black bars) and LOX (gray bars) melanoma cells were transfected with IKKα-directed or control siRNA for 48 hours, followed by incubation with 0.5 µM doxorubicin for 24 hours. Subsequently, the generation of histone-bound DNA fragments was measured by ELISA. The measured values were normalized to untreated cells. The values shown represent the average of three independent experiments each performed in triplicate (±SEM).

The experiments described thus far suggested that IKKα has a very limited, if any, influence on anti-tumoral treatments in the melanoma cells used in this study. In particular, doxorubicin-induced activation of NF-κB and, consecutively, regulation of NF-κB-dependent gene products or cellular functions were only marginally or not at all altered by inhibition of IKKα. The

latter notion applied to both small-molecule- and siRNA-based inhibition of IKKα.

7.8 Doxorubicin-induced NF-κB activity is mediated by ATM

It has been shown, that doxorubicin induces DNA double strand breaks and that the nuclear protein kinase ataxia telangiectasia mutated (ATM) becomes auto-phosphorylated as a consequence of these DNA-double strand breaks (Kurz et al. 2004; Wu et al. 2006). In the nucleus, activated ATM phosphorylates IKKγ in an assembly with PARP1 and PIASγ. This leads to cytoplasmic export of an IKKγ-ATM complex. The regulatory IKK subunit, IKKγ, activates the cytoplasmic IKK complex and thus the NF-κB pathway becomes activated in response to double strand breaks (Stilmann et al. 2009).

To address whether doxorubicin-induced NF-κB activity in melanoma cells is influenced by ATM, cells were treated with a combination of doxorubicin and the ATM-inhibitor, KU55933. Indeed, the measurement of the NF-κB dependent luciferase synthesis revealed that the combined treatment with doxorubicin and KU55933 completely abolished the doxorubicin-induced NF-κB activity to the level of vehicle treated cells ($p<0.003$; Figure 30).

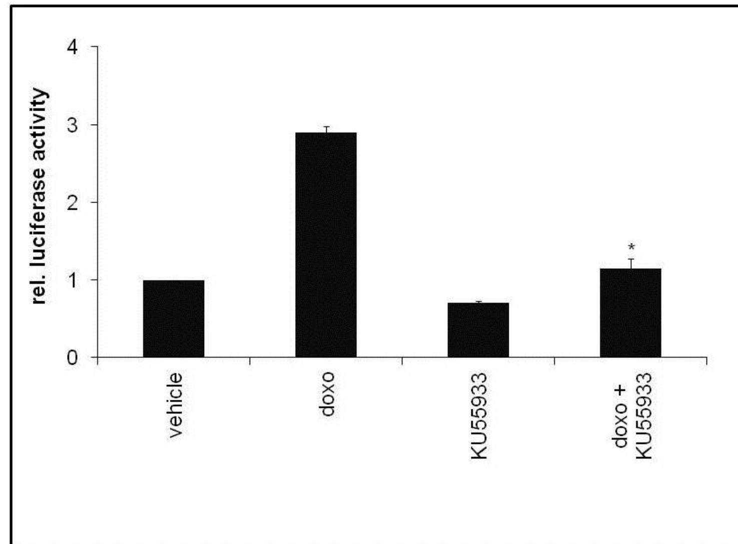


Figure 30: Doxorubicin induced activation of NF-κB is abrogated by the ATM inhibitor, KU55933.

The melanoma cell line A375 was transfected with the plasmids for NF-κB dependent luciferase assay for 24 hours, followed by treatment with 1 µM doxorubicin, 10 µM KU55933 or the combination of both for 8 hours. Subsequently, NF-κB-dependent luciferase synthesis was measured. The values were normalized to vehicle-treated cells. The values shown represent the averages of three independent experiments, each performed in triplicate (±SEM). * indicates $p < 0.0003$ compared to treatment with doxorubicin alone.

7.9 Induction of double strand breaks and phosphorylation of ATM by temozolomide and cisplatin did not result in NF-κB activation

Other chemotherapeutic drugs, such as temozolomide and cisplatin, are also known to induce double strand breaks. However, compared to doxorubicin these chemotherapeutics do not directly induce DNA-double strand breaks but rather modify the DNA strands through modulation of protein-DNA interactions (Sorenson and Eastman 1988; Agarwala and Kirkwood 2000). The phosphorylation of histone H2AX at serine 139 (called γH2AX) was described as a sensitive marker for cellular responses to DNA double strand breaks (Rogakou et al. 1998). In order to investigate whether exposure to temozolomide or cisplatin for longer periods of time would induce double strand breaks in melanoma cells, the generation of γH2AX was detected by

immunofluorescence staining after 8, 24, 48 and 72 hours, respectively. As expected, the number of dead cells increased with longer exposure times with either of the compounds at concentrations determined in preliminary dose-response experiments. In the case of 0.5 mM temozolomide, the number of cells available after 72 hours was large enough for experimental investigations, while the concentration of cisplatin had to be reduced to 1 µM to maintain an amount of cells sufficient for further analyses. In temozolomide-treated cells, the density of the γH2AX signal increased during the first 48 hours, thereafter the γH2AX signal intensity decreased. Likewise, the numbers of cell nuclei with γH2AX signals increased in cisplatin treated cells during the first 48 hours, and decreased again after that time (Figure 31). The temozolomide and cisplatin induced γH2AX signals were weaker compared to doxorubicin-induced signals (Figure 31). These observations suggested that temozolomide and cisplatin induce double strand breaks but later and weaker as doxorubicin.

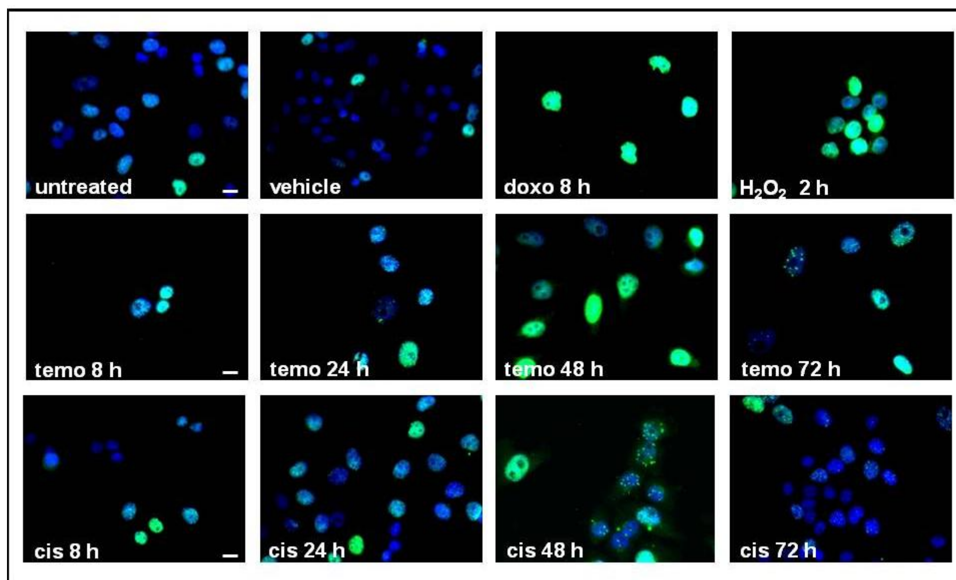


Figure 31: Temozolomide and cisplatin induce H2AX phosphorylation in melanoma cells later and less pronounced compared to doxorubicin.

A375 melanoma cells were treated with doxorubicin for 8 hours, H_2O_2 for 2 hours or temozolomide or cisplatin for 8, 24, 48 or 72 hours, respectively. The cells are fixed and probed with anti-γH2AX antibody followed by a FITC-conjugated secondary antibody. The immunofluorescence of γH2AX detection of each treatment was photographed using identical exposure times. The pictures are representative for two independent experiments showing similar results. Scale bars = 10 µm.

ATM becomes activated in response to DNA double strand breaks. It then phosphorylates various substrates, including H2AX, which are thought to be involved in DNA damage response (Burma et al. 2001; Shiloh 2006). As shown above, both temozolomide and cisplatin treatment induced the phosphorylation of H2AX (Figure 32). These notions were the rationale for the following investigations addressing the question whether γH2AX activation in human melanoma cells by doxorubicin, temozolomide or cisplatin, respectively, was associated with ATM activation. In line with the results of the above immunofluorescence experiments, Western blot analysis revealed that doxorubicin-treated cells showed the highest amount of γH2AX. However, treatment of melanoma cells with temozolomide or cisplatin for 48 or 72 hours, respectively, also increased the phosphorylation of H2AX, albeit at markedly lower levels as compared to doxorubicin (Figure 32). The unphosphorylated ATM was reduced in doxorubicin-treated cells and also in cells, which were treated for 24 hours with temozolomide or cisplatin, respectively. Compared to untreated or vehicle-treated cells, the content of phosphorylated ATM was elevated in cells exposed to the chemotherapeutics, again with the strongest signals detected in doxorubicin-treated cells (Figure 32).

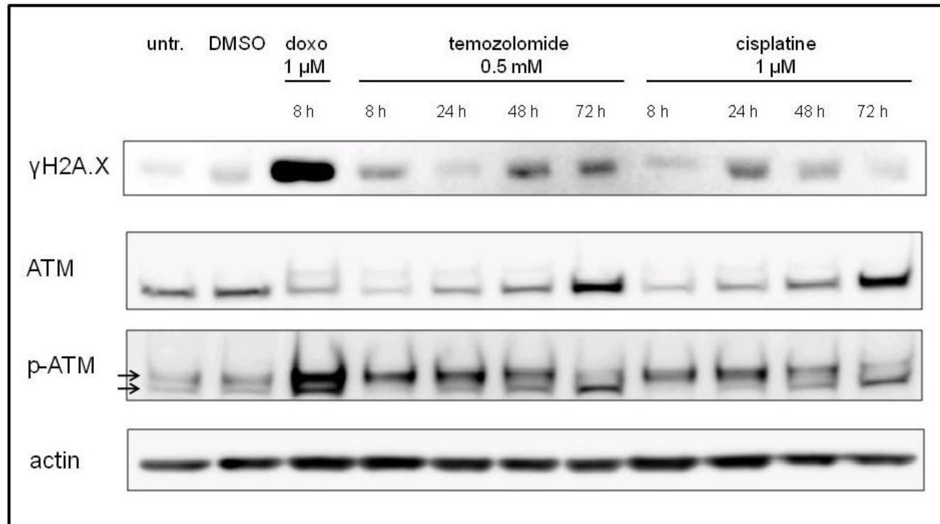


Figure 32: Incubation of melanoma cells with temozolomide or cisplatin leads to phosphorylation of H2AX and ATM, respectively, and both effects are not as strong as those induced by doxorubicin.

A375 melanoma cells were incubated with 1 µM doxorubicin, 0.5 mM temozolomide or 1 µM cisplatin, respectively, as indicated. Whole cell lysates were assessed by Western blot for γH2AX, ATM and phosphorylated ATM (p-ATM). The antibody directed against p-ATM revealed two bands: We speculate that the lower signal represents unphosphorylated ATM, whereas the upper band corresponds to p-ATM. The highest content of p-ATM was seen in doxorubicin-exposed cells. Actin was detected as a loading control. The Western blot shown is representative for two independent experiments showing similar results.

Both temozolomide- and cisplatin-treated melanoma cells feature an increased amount of double strand breaks and phosphorylated ATM (Figure 31 and 32). It is known that double strand breaks can activate ATM as well as NF-κB (Kurz et al. 2004; Wu et al. 2006). Interestingly enough, when the NF-κB activation in A375 melanoma cells was investigated after treatment with temozolomide or cisplatin for 72 hours, the NF-κB activity was not affected (Figure 33), although clear induction of DNA double strand breaks and ATM activation had been demonstrated (Figure 32).

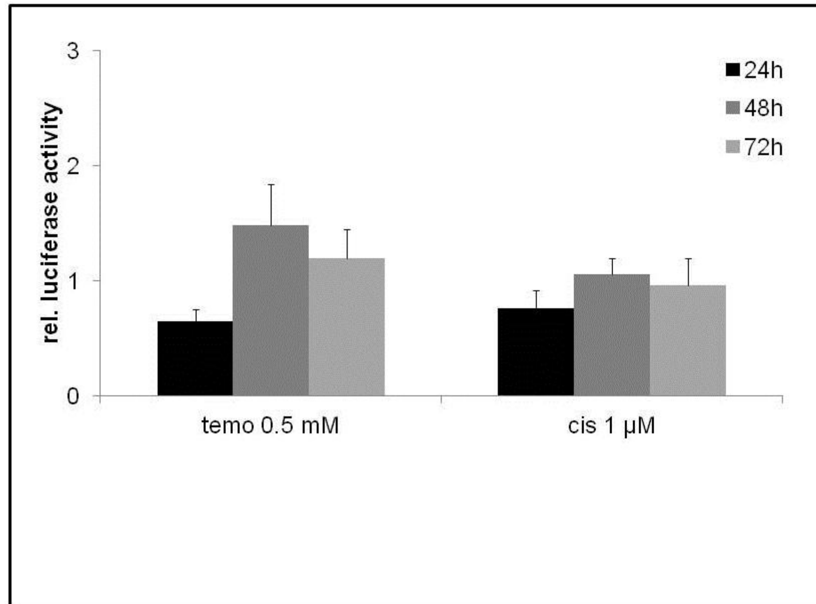


Figure 33: The exposure to temozolomide and cisplatin for 72 hours did not induce NF-κB activity in melanoma cells.

A375 melanoma cells were transfected with the plasmids for the NF-κB-dependent luciferase assay for 24 hours, followed by treatment with 0.5 mM temozolomide or 1 µM cisplatin, respectively. The cells were cultured for 24, 48 or 72 hours and then luciferase activity was determined. The values were normalized to vehicle-treated cells. The values shown depict the average of three independent experiments (±SEM).

7.10 NF-κB inhibition by blocking ATM activity increases doxorubicin-induced apoptosis

The starting point of this thesis has been the observation that treatment of melanoma cells with doxorubicin induced NF-κB activation, followed by enhanced transcription of NF-κB-regulated genes, which are thought to be involved in tumor progression (Figures 4 and 9). Moreover, inhibition of NF-κB through the IKKβ inhibitor, KINK-1, abrogated the doxorubicin-induced NF-κB activation as well as the downstream increment of NF-κB related gene transcription. The combination of doxorubicin and KINK-1 had an additive or synergistic effect on cellular apoptosis (Figures 9, 11 and 12). Extending these findings, inhibition of constitutive NF-κB activity in melanoma cells treated with

the ATM inhibitor, KU55933, was reduced by approximately 40 % as compared to vehicle-treated control cells ($p<0.0001$; Figure 34).

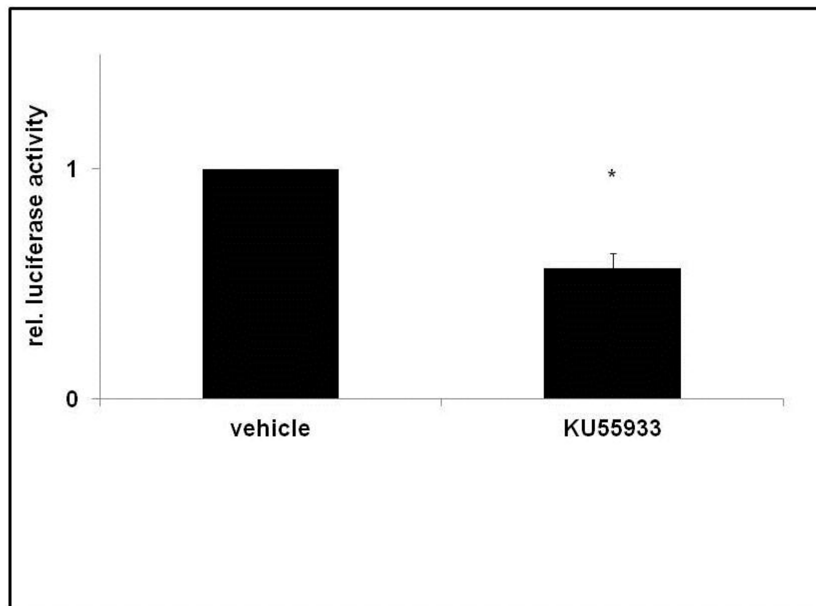


Figure 34: Constitutive NF-κB activity of melanoma cells was inhibited by KU55933, a specific inhibitor of ATM.

A375 melanoma cells were transfected with plasmids for the NF-κB-dependent luciferase assay followed by treatment with 10 µM KU55933 for 8 hours. NF-κB-dependent luciferase assay were performed. The measured values were normalized to vehicle treated cells. The figure shows the average of six independent experiments, each performed in triplicate (±SEM). * indicates $p< 0.0001$

In order to investigate whether the impairment of NF-κB activation elicited by the ATM inhibitor, KU55933, would increase doxorubicin-induced apoptosis, melanoma cells were exposed for 24 hours to doxorubicin alone or to the combination of doxorubicin and KU55933. Subsequently, the generation of histone-bound DNA fragments was measured by ELISA. Indeed, NF-κB inhibition by KU55933 increased the doxorubicin-induced apoptosis by approximately 2-fold in both melanoma cell lines compared to cells treated with doxorubicin alone (Figure 35).

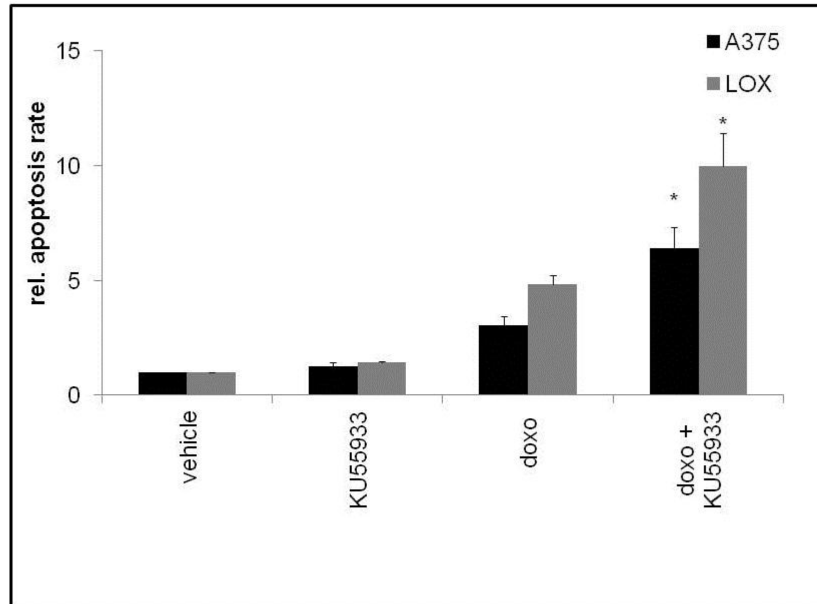


Figure 35: NF-κB inhibition by the ATM inhibitor, KU55933, increases doxorubicin-induced apoptosis in melanoma cells.

A375 (black bar) and LOX (gray bar) melanoma cells were subjected to single treatment with 0.5 µM doxorubicin, 10 µM KU-55933 or to combined treatment with both agents for 24 hours. Apoptosis was measured by ELISA detecting the generation of histone-bound DNA fragments. The measured values were normalized to untreated cells. The figure shows the average of three independent experiments, each performed in triplicate (±SEM). * indicates $p < 0.01$

Finally, apoptosis of melanoma cells was examined in response to treatment with temozolomide or cisplatin in combination with the ATM inhibitor, KU55933. While treatment with either of the cytostatics alone led to an approximately 6-fold increase of apoptosis, the additional exposure to the ATM inhibitor did not result in further enhancement (Figure 36).

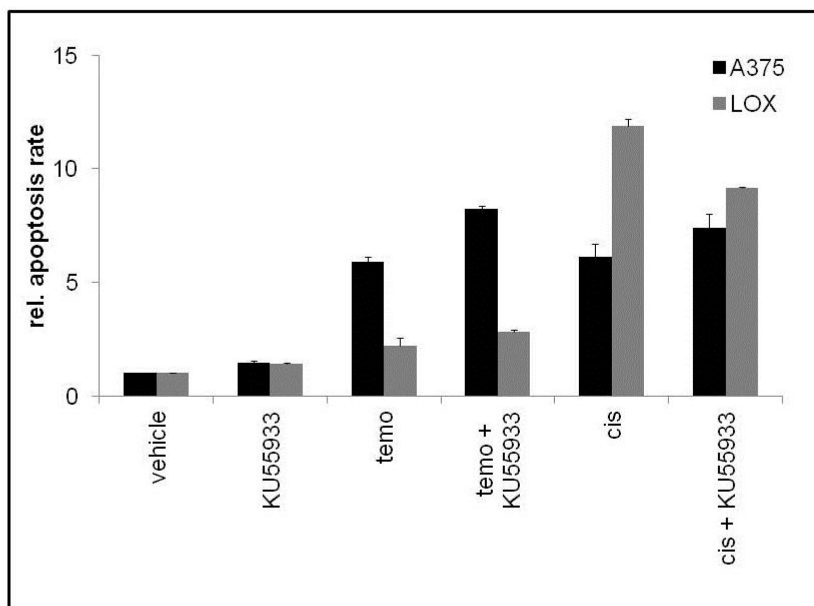


Figure 36: ATM inhibition by KU55933 did not increase temozolomide- or cisplatin-induced apoptosis in melanoma cells.

A375 and LOX melanoma cells were exposed to 0.5 µM temozolomide, 5 µM cisplatin, 10 µM KU55933 or to combined treatment as indicated for 24 hours. Apoptosis was determined by ELISA measuring the generation of histone-bound DNA fragments. The values are normalized to the apoptosis of vehicle-treated cells. The values shown are averages of three independent experiments, each performed in triplicate (±SEM).

Overall, ATM inhibition by a small molecule inhibitor led to abrogation of doxorubicin-induced NF-κB activity in two different melanoma cell lines. As a consequence, it is conceivable that inhibition of ATM enhanced the pro-apoptotic effect exerted by NF-κB activating chemotherapeutics, such as doxorubicin. In contrast, apoptosis induced by chemotherapeutics which not activate the NF-κB signaling pathway was not significantly affected by ATM inhibition

8 Discussion

Melanoma is an aggressive malignancy, especially in metastasized stages. In fact, it is the deadliest skin disease and its worldwide incidence is rising continuously. Unfortunately, advanced melanoma is almost universally resistant to irradiation and chemotherapeutic treatment (Bene et al. 2008). The year 2011, after decades of factual stagnation, has seen the approval of two novel therapeutic drugs, both of which have shown benefits for patients in terms of survival, Vemurafenib (a B-RAF inhibitor, trade name Zelboraf®) and Ipilimumab (a T cell activator, trade name Yervoy®) (Eggermont and Robert 2011). Notwithstanding the euphoria that accompanied the advent of these new compounds, their impact on overall survival of the patients is still only a few months. Moreover, treatment with these compounds is not suitable for all patients and the development of resistance mechanisms has been described (Nazarian et al. 2010). Thus, a better understanding of the melanoma resistance mechanisms is still desirable, a notion that is also highly relevant as far as individualized therapies are concerned.

Chemotherapeutics, NF-κB activation and implications for chemoresistance of melanoma cells

This work has demonstrated that amongst a panel of cytostatics with different modes of action only doxorubicin, an anthracycline drug, had the capacity to activate NF-κB in several melanoma cell lines. Likewise, NF-κB-dependent transcription of gene products, which are thought to be involved in tumor progression, was enhanced by doxorubicin. Notably, the most pronounced activation of NF-κB was measured at a concentration of 1 μM doxorubicin, i. e. a concentration typically reached in the serum of doxorubicin-treated patients (Palm et al. 2001). While it is certainly curious to speculate about potential mechanisms for this peak activity, the most likely explanation appears to be the direct cytotoxicity exerted by doxorubicin at higher

concentrations, which may lead to a general down-modulation of protein transcription. It is, however, also conceivable that other, more specific mechanisms contribute to the biphasic NF-κB-related response of melanoma cells to doxorubicin. A possible interpretation can be discerned indirectly from neurobiological experiments, where a similar correlation of doxorubicin concentration and NF-κB activity has been described in rat cortical neurons. In these experiments, the generation of ROS was also dependent of doxorubicin concentration, just like NF-κB activity (Lopes et al. 2011). Given that the generation of ROS is necessary for NF-κB activation, such interactions could contribute to the variable doxorubicin-induced NF-κB activity at different concentrations (Wuerzberger-Davis et al. 2007). Another possibility is that the cellular effects of doxorubicin at higher concentrations modulate additional signaling pathways, such as the E2F1 transcription factor or the A20 ubiquitin editing complex, which may impact indirectly on NF-κB activity (Tanaka et al. 2002; Shembade et al. 2011).

An important question that arose from the results of this study was why NF-κB was activated preferentially by doxorubicin, but not by a number of other cytostatics. Given that considerable efforts have been invested in the recent past into the clarification of the mode of action of many anti-tumoral drugs, several hypotheses, which are not mutually exclusive, can be delineated to approach this question:

It is known that activation of NF-κB by doxorubicin requires both the induction of double strand breaks (DSB) and the generation of reactive oxygen species (ROS) (Wuerzberger-Davis et al. 2007). In this way poly(ADP-ribose) polymerase-1 (PARP-1) becomes activated by DNA damage to assemble a signalosome together with protein inhibitor of activated STAT γ (PIASγ), ATM and IKKγ (Stilmann et al. 2009). In this constellation, IKKγ becomes phosphorylated at Ser-85 by ATM and SUMOylated by PIASγ at Lys-285. The ATM/IKKγ complex translocates to the cytoplasm (Wu et al. 2006; Wu et al. 2010). Subsequently, the cytoplasmic IKKα/IKKβ/IKKγ complex becomes

activated due to transfer of ATM activity to TAK1 by ubiquitination events (Hadian and Krappmann 2011). Based on previous studies, it is conceivable that other chemotherapeutics do not activate NF-κB due to a lack of this “nuclear-cytoplasm” pathway. Indeed, temozolomide generates only a moderate amount of ROS necessary for SUMOylation of IKKγ (Wuerzberger-Davis et al. 2007). As a consequence, the entire cascade is hampered. Another explanation appears to apply to cisplatin, which preferentially activates ATR instead of ATM. Interestingly, ATR can prevent the phosphorylation of IKKγ at Ser-85 and, presumably, block the downstream activation of NF-κB (Huang et al. 2008; Wu and Miyamoto 2008; Tanida et al. 2012). Furthermore, vincristin can inhibit PARP-1 activity, and 5-fluorouracil appears to affect RNA metabolism rather than DNA metabolism (Longley et al. 2003; Chatterjee et al. 2008). Thus, in melanoma the development of chemoresistance against temozolomide, cisplatin, vincristine and 5-fluorouracil is not mediated by NF-κB activation. The mechanisms how these chemotherapeutic agents induce resistance are also a matter of ongoing research, for example it has been shown that cisplatin resistance can be induced by reduction of an ERK inactivating phosphatase (DUSP6) followed by ERK dependent increase of the DNA repair proteins, ERCCC1 and XPF (Li and Melton 2011).

Of note, blockade of NF-κB by KINK-1, an inhibitor of IKKβ, but not by the novel IKKα inhibitor, BAY32-5915, completely abrogated doxorubicin-induced NF-κB activity and increased the apoptotic response of melanoma cells. In contrast, NF-κB inhibition failed to increase cisplatin-induced apoptosis, a notion that is consistent with the lack of NF-κB activation by cisplatin. However, although cisplatin-induced apoptosis in melanoma cells was not enhanced by combination with NF-κB inhibition, such combinatorial approaches may succeed in other types of cancer, such as human cervical cancer (Venkatraman et al. 2005; Pletz et al. 2012). It appears, therefore, that certain chemotherapeutics may act variably on different cell types, thus precluding generalized predictions.

In any case, the results of this study suggest that inhibiting NF-κB can overcome resistance of melanoma cells to some chemotherapeutics, whose activity comprises the induction of NF-κB as a part of drug-induced chemoresistance. Based on this hypothesis, it is reasonable to assume that the efficacy of such drugs, even at lower concentrations, will be maintained by this kind of additional strategies (Das and White 1997; Shattuck-Brandt and Richmond 1997; Wang et al. 1999; Amiri and Richmond 2005). Lower doses of cytostatic drugs are predicted to possess a superior safety profile, which in case of doxorubicin may mean reduction of the risk of doxorubicin-mediated cardiomyopathy, a feared and oftentimes dose-limiting side effect (Chatterjee et al. 2010).

IKKα and IKKβ and their role as potential targets in melanoma therapy

Given the importance of NF-κB for tumor progression and chemoresistance, it is not surprising that numerous studies have shown that inhibition of NF-κB activity may aid the susceptibility of melanoma cells to chemotherapeutic treatment (Huang et al. 2000; Amiri et al. 2004; Schön et al. 2008; Amschler et al. 2010; Pletz et al. 2012). However, there are several possible ways to interfere with NF-κB activity, and it is conceivable that tampering with one given mechanism might still achieve a therapeutic response once the tumor cells have developed resistance against others (Amschler et al. 2010). The latter notion gave rise to the investigations of blocking IKKα, since inhibition of IKKβ as well as inhibition of the proteasome have yielded promising results already in several preclinical studies.

The first experiments in this thesis comprised the use of a novel small-molecule IKKα inhibitor (BAY32-5915), whose specificity had been demonstrated in large cell-free assays by researchers at Bayer HealthCare (Pletz et al. 2012). As it turned out, no clear-cut impact of the IKKα inhibitor BAY32-5915 on melanoma cells could be detected, despite rather extensive

attempts in various experimental systems. Not only did a novel small-molecule inhibitor of IKKα fail to affect the activity of NF-κB in the melanoma cell lines tested, silencing of IKKα, too, had only moderate, if any, effects on NF-κB activity as well as downstream gene transcription. Likewise, depletion of IKKα did not affect the synthesis of several apoptosis-related proteins or cellular functions such as migration, proliferation or apoptosis induction by doxorubicin.

Thus, the next interesting question that arose from this study was why IKKβ inhibition was so much more effective than IKKα inhibition with respect to blocking NF-κB activity and increasing the susceptibility of melanoma cells for chemotherapy. The failure of BAY32-5915 to inhibit NF-κB and to enhance the susceptibility of melanoma cells to chemotherapy, which is in stark contrast to the effect seen with the IKKβ inhibitor, KINK-1, as well as the subsequent lines of experiments gave rise to a number of notions and hypotheses, which are not mutually exclusive and which will be discussed in the following:

The expression levels of the IKK subunits, IKKα and IKKβ, varied considerably between different human melanoma cell lines, although the ratio of the two appeared to be rather constant. However, no correlation with the constitutive or stimulated activity of NF-κB was found (Amschler et al. 2010). While an easy explanation for this apparent discrepancy cannot be discerned from the literature, it is conceivable that other factors, which are not IKK-related, contribute to the activation status of NF-κB. For example, IκBα degradation occurs by doxorubicin treatment in IKKα/β knockout MEFs and by Casein kinase II phosphorylation at the C-terminal site under UV-mediated cell stress (Kato et al. 2003; Tergaonkar et al. 2003). Indeed, while the classical or the alternative activation by IKK complexes (IKKα/IKKβ/IKKγ-heterotrimer or IKKα/IKKα-homodimer, respectively) certainly is the best-studied pathway, some previous studies have demonstrated that certain cell types may possess active NF-κB without any external stimuli (Karin and Ben-Neriah 2000; Brivanlou and Darnell 2002). Further, post-translational modification of NF-κB may influence the transcriptional activity; for example, phosphorylation of RelA

at Ser-276 enhanced its DNA binding and interaction with the co-activator CBP/p300 (Perkins 2006). Moreover, it is ultimately unclear which, if any, other isoforms of NF-κB – besides the well-studied p50/p65 heterodimer – contribute to the net activity of NF-κB in melanoma cells. Therefore, it is conceivable that some of such other isoforms may become activated independent of the IKK complex and of IKKα in particular.

The results of this thesis are in accordance with the literature and support the theory that inhibition of IKKβ, but not IKKα, is a potential way to overcome chemoresistance in melanoma. However, the differential responses of melanoma cells to IKKα versus IKKβ inhibition still remain somewhat enigmatic. In accordance with our results, it was shown that the IKKβ kinase has a 50 - 60-fold higher activity to phosphorylate IκBα compared to IKKα. In addition, IKKα is thought to be a regulating rather than a catalyzing factor for the high intrinsic IKKβ activity (Li et al. 1998; O'Mahony et al. 2000; Yamamoto et al. 2000). In contrast, other examinations revealed that the depletion of IKKα reduced NF-κB activity and increased apoptosis similar to IKKβ reduction (Bednarski et al. 2008; Adli et al. 2010). Furthermore, IKKα can free p65 by phosphorylation at Ser-536 without degradation of IκBα, and after TNFα stimulation, IKKα seemed to be responsible for optimal gene transcription by modification of histone H3 and CREB binding protein (Anest et al. 2003; Yamamoto et al. 2003; Anest et al. 2004; Huang et al. 2007). Additionally, IKKα is crucial for doxorubicin-induced NF-κB activation in sarcoma cells (Bednarski et al. 2008). Again, these apparently differential activities may be cell type-specific.

Along this line, IKKα is an important regulator of differentiation in B cells and keratinocytes, lack of IKKα leads to uncontrolled proliferation. In B cells, IKKα mediates the alternative pathway of NF-κB activation and in keratinocytes it appears to exert some functions independent of NF-κB (Senftleben et al. 2001; Liu et al. 2006; Zhu et al. 2007). These findings suggested that the activity of IKKα may differ between cell types.

Recent studies even suggested that IKKα has a NF-κB inhibiting function and that it also acts as tumor suppressor (Kwak et al. 2011; Shembade et al. 2011). According to these publications, IKKα is involved in the termination of NF-κB activity through phosphorylation of TAX1-binding protein1 (TAX1BP1) followed by activation of the A20 ubiquitin editing complex, which antagonizes TRAF6 and TRAF2 for further activation of the IKK complex (Shembade et al. 2011). Furthermore, IKKα phosphorylated nuclear cyclin D1 for nuclear export and subsequent cytoplasmic degradation in MEFs (Kwak et al. 2011). In accordance to these recent insights, it might be possible that IKKα becomes inactivated in melanoma cells. If this assumption is correct, this could explain, at least in part, why depletion of IKKα did not affect the melanoma cells. However, there is currently no experimental data in support of this hypothesis.

ATM functions and implications for chemoresistance of melanoma cells

As shown in this study, blocking of ATM by a small molecule inhibitor inhibited NF-κB activity and increased doxorubicin-induced apoptosis in melanoma cells. Therefore, another question drawn from this study that appeared worthwhile discussing was if and how ATM signaling could cross-talk with the NF-κB pathway and what this could mean for chemoresistance in melanoma cells:

ATM was necessary for the activation of NF-κB by doxorubicin; in turn, inhibition of ATM reduced doxorubicin-induced NF-κB activity and increased the induction of apoptosis. In response to DSB, ATM activates a plethora of substrates, including DNA repair proteins, which can also be activated directly by NF-κB (Volcic et al. 2012). As proposed recently, the p65 subunit of NF-κB interacts directly with the CtIP-BRAC1 complex for stabilization. This interaction advances DSB repair and promotes chemoresistance against doxorubicin in breast cancer cells. Thus, ATM inhibition blocks DNA repair by ATM and also by NF-κB. Furthermore, ATM inhibition interferes with additional pathways implicated in tumor progression. Along this line, γ-irradiation of melanoma cells

in the presence of KU55933 increases the expression of death receptor 5 (DR5) by inhibiting the STAT3 transcription factor. As a consequence, TRAIL induced apoptosis becomes enhanced (Ivanov et al. 2009). The STAT3 signaling pathway is constitutively active in many types of cancer including melanoma, and its activation appears to correlate with poor prognosis (Niu et al. 2002; Kusaba et al. 2006). The constitutive activity of STAT3 constitutes another important pathway of melanoma survival and progression (Niu et al. 2002). Additionally, blocking of ATM may suppress the proliferation of cancer cells and enhance rampamycin-induced apoptosis through the prevention of AKT phosphorylation. Notably, 60 - 70 % of sporadic melanomas exhibit increased activity of AKT (Madhunapantula and Robertson 2009). Therefore, the inhibition of ATM in melanoma cells could be advantageous compared to NF-κB inhibition alone.

Taken together, it is conceivable that modulating ATM functions by small molecule inhibitors will be beneficial in combination with γ-irradiation or doxorubicin treatment, because such combinatorial treatments target more than one survival pathway of melanoma cells. However, this hypothesis needs to be tested in future studies, e. g. by investigating the combinations of doxorubicin with KINK-1 or KU55933. Moreover, the combination of doxorubicin with KU55933 (or related compounds) could be assessed regarding their effects on cardiomyocytes; such experiments could be reasonable with respect to doxorubicin-mediated cardiotoxicity (Wang et al. 2004). One may speculate that toxicity in cardiomyocytes can be prevented or ameliorated by additional inhibition of ATM.

In summary, doxorubicin-induced activation of NF-κB appears to contribute to chemoresistance of melanoma cells against this drug. Moreover, inhibition of either ATM or IKKβ constitutes two potential ways to overcome such chemoresistance to doxorubicin. Mechanisms of chemoresistance against other cytostatics, such as temozolomide or cisplatin, are different from that against

doxorubicin. The findings of this study highlight the rationale for intelligent combinatorial treatment strategies, potentially including inhibition of IKKβ or ATM, as a part of individualized therapies of melanoma patients.

9. Summary and conclusion

In this study we showed that doxorubicin led to a profound activation of NF-κB in two different melanoma cell lines, while temozolomide, cisplatin, vincristine and 5'fluorouracil did not activate this pathway. Notably, the strongest NF-κB activation and NF-κB-dependent gene transcription was detected at a concentration of 1 μM doxorubicin corresponding to the concentration reached when malignant tumors are treated *in vivo*. Further, doxorubicin-induced NF-κB activity was inhibited by applying small molecules blocking either of the two major kinases of the NF-κB pathway, namely IKKα and IKKβ. The novel IKKα inhibitor (BAY32-5915) was identified from a large library for specific IKKα inhibition and the IKKβ inhibitor (KINK-1) was previously described in our group. The IKKβ inhibitor completely abrogated doxorubicin-induced NF-κB activity, whereas the IKKα inhibitor did not affect the induced activity. Furthermore, doxorubicin combined with the IKKβ inhibitor, but not with the IKKα inhibitor, increased the apoptotic response of melanoma cells. Complementary, while silencing of IKKβ by siRNA notably reduced doxorubicin-induced NF-κB activity, silencing of IKKα had only a moderate effect on NF-κB activation by doxorubicin and downstream gene transcription. Additionally, reduction of IKKα by siRNA did not affect the synthesis of apoptosis-related proteins or cellular functions such as migration, proliferation or apoptosis. Accordingly, IKKα reduction did not increase the susceptibility of melanoma cells to doxorubicin. Moreover, we showed that inhibition of ATM, by small molecule inhibitors, abrogated doxorubicin-induced NF-κB activity and also increased susceptibility of melanoma cells to doxorubicin, but not to temozolomide or cisplatin.

In search for improved therapeutic strategies, our study revealed that targeting IKKα for NF-κB inhibition to overcome chemoresistance in melanoma is not as effective as IKKβ inhibition. However, recent publications report about tumor suppressor function of IKKα; therefore the progression of melanoma cells overexpressing IKKα should be addressed. As ATM inhibition is equally

efficiently as IKKβ inhibition to overcome doxorubicin-induced chemoresistance, ATM inhibition might be more efficient in combinational treatment of melanoma because it additionally affects several survival pathways besides NF-κB. Our findings suggest that intelligent combined treatments including inhibition of IKKβ or ATM can be a promising strategy for therapy of metastasized melanoma.

10 Supplements

10.1 The IKKα Inhibitor, BAY32-5915

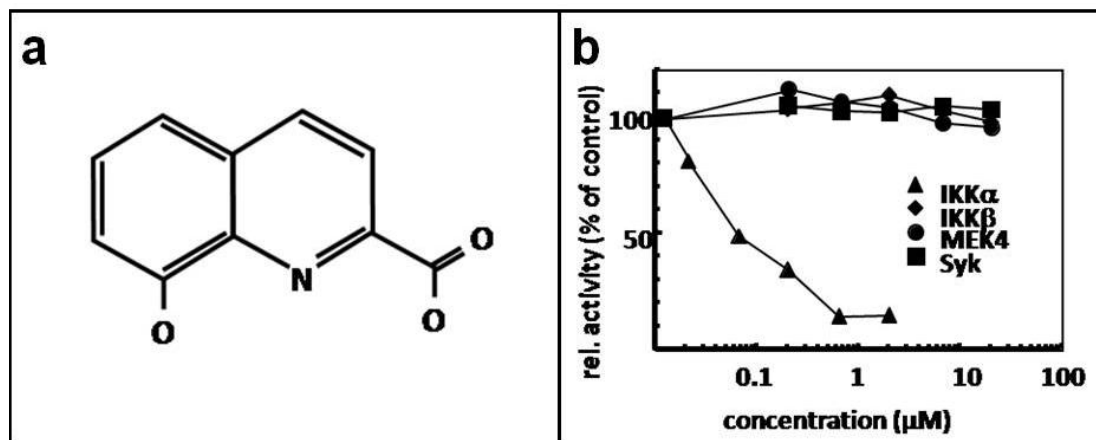


Figure 37: Chemical structure and activity of the novel compound 8-hydroxyquinoline-2-carboxylic acid (BAY32-5915).

(a) The chemical structur of the 8- hydroxyquinoline-2-carboxylic acid (BAY32-5915). (b) The activity of 8- hydroxyquinoline-2-carboxylic acid (BAY32-5915) was assessed in standardized cell free in vitro kinase assays on IKKb (diamonds; substrate: GST-IkBa (1–54), 0.2 IM; ATP, 5 IM), IKKa (triangles; substrate: full length GST-IkBa, 0.2 IM; ATP, 0.2 IM), MEK4 (circles; substrate: GST-SAPKaKN, 0.2 IM; ATP, 1.5 IM) or Syk (squares; substrate: activation loop peptide, 2.1 IM; ATP, 30 IM). The values depict the relative activity of the enzymes at the indicated concentrations of the compound. The experiment shown is representative of four independent experiments showing similar results. Reference inhibitors were used in all kinase assays as positive controls of assay quality. These reference compounds were BAY61-3606, a Syk inhibitor with an IC50 of 13 nM, BAY66-1775, a MEK4 inhibitor with an IC50 of 280 nM as well as KINK-1, a selective IKKb inhibitor with an IC50 of 2–4 nM. (Figure from (Pletz et al. 2012); data provided by Dr. K. Ziegelbauer, Bayer HealthCare, Wuppertal).

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